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(54) Title: HUMAN HOMOLOG OF THE DROSOPHILA PROTEIN "FUSED"

(57) Abstract

The present invention relates to nucleotide sequences, including expressed sequence tags (ESTs), oligonucleotide probes, polypeptides, vectors and host cells expressing, immunoadhesins, agonists and antagonists to human and vertebrate *fused*.

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HUMAN HOMOLOG OF THE DROSOPHILA PROTEIN "FUSED"

FIELD OF THE INVENTION

The present invention relates generally to signaling molecules, specifically to signaling and mediator molecules in the hedgehog (*Hh*) cascade which are involved in cell proliferation and differentiation.

BACKGROUND OF THE INVENTION

Development of multicellular organisms depends, at least in part, on mechanisms which specify, direct or maintain positional information to pattern cells, tissues, or organs. Various secreted signaling molecules, such as members of the transforming growth factor-beta (TGF-β), Wnt, fibroblast growth factors and hedgehog families have been associated with patterning activity of different cells and structures in *Drosophila* as well as in vertebrates. Perrimon, *Cell*: 80: 517-520 (1995).

Hedgehog (*Hh*) was first identified as a segment-polarity gene by a genetic screen *in Drosophila melanogaster*, Nusslein-Volhard *et al., Roux. Arch. Dev. Biol.* 193: 267-282 (1984), that plays a wide variety of developmental functions. Perrimon, *supra*. Although only one Drosophila *Hh* gene has been identified, three mammalian *Hh* homologues have been isolated: Sonic *Hh (SHh)*, Desert *Hh (DHh)* and *Indian Hh (IHh)*, Echelard *et al., Cell* 75: 1417-30 (1993); Riddle *et al., Cell* 75: 1401-16 (1993). *SHh* is expressed at high level in the notochord and floor plate of developing vertebrate embryos. In vitro explant assays as well as ectopic expression of *SHh* in transgenic animals show that *SHh* plays a key role in neuronal tube patterning, Echelard *et al., supra.*, Krauss *et al., Cell* 75, 1431-44 (1993), Riddle *et al., Cell* 75: 1401-16 (1993), Roelink *et al, Cell* 81: 445-55 (1995). In vitro explant assays as well as ectopic expression of SHh in transgenic animals show that SHh plays a key role in neural tube patterning, Echelard *et al.* (1993), *supra.*; Ericson *et al., Cell* 81: 747-56 (1995); Marti *et al., Nature* 375: 322-5 (1995); Roelink et al. (1995), *supra*; Hynes *et al., Neuron* 19: 15-26 (1997). *Hh* also plays a role in the development of limbs (Krauss *et al., Cell* 75: 1431-44 (1993); Laufer *et al., Cell* 79, 993-1003 (1994)), somites (Fan and Tessier-Lavigne, *Cell* 79, 1175-86 (1994); Johnson *et al., Cell* 79: 1165-73 (1994)), lungs (Bellusci *et al., Develop.* 124: 53-63 (1997) and skin (Oro *et al., Science* 276: 817-21 (1997). Likewise, *IHh* and *DHh* are involved in bone, gut and germinal cell development, Apelqvist *et al., Curr. Biol.* 7: 801-4 (1997); Bellusci *et al., Development.* 124: 53-63 (1997); Bitgood *et al., Curr. Biol.* 6: 298-304 (1996); Roberts *et al., Development* 121: 3163-74 (1995). *SHh* knockout mice further strengthened the notion that *SHh* is critical to many aspect of vertebrate development, Chiang *et al., Nature* 383: 407-13 (1996). These mice show defects in midline structures such as the notochord and the floor plate, absence of ventral cell types in neural tube, absence of distal limb structures, cyclopia, and absence of the spinal column and most of the ribs.

At the cell surface, the Hh signals is thought to be relayed by the 12 transmembrane domain protein *Patched* (*Ptch*) [Hooper and Scott, *Cell* 59: 751-65 (1989); Nakano *et al., Nature* 341: 508-13 (1989)] and the G-protein coupled like receptor Smoothened (*Smo*) [Alcedo et al., Cell 86: 221-232 (1996); van den Heuvel and Ingham, Nature 382: 547-551 (1996)]. Both genetic and biochemical evidence support a receptor model where *Ptch* and *Smo* are part of a multicomponent receptor complex, Chen and Struhl, *Cell* 87: 553-63 (1996); Marigo *et al., Nature* 384: 176-9 (1996); Stone *et al., Nature* 384: 129-34 (1996). Upon

binding of *Hh* to *Ptch*, the normal inhibitory effect of *Ptch* on *Smo* is relieved, allowing *Smo* to transduce the *Hh* signal across the plasma membrane. Loss of function mutations in the *Ptch* gene have been identified in patients with the basal cell nevus syndrome (BCNS), a hereditary disease characterized by multiple basal cell carcinomas (BCCs). Disfunctional *Ptch* gene mutations have also been associated with a large percentage of sporadic basal cell carcinoma tumors, Chidambaram *et al.*, *Cancer Research* 56: 4599-601 (1996); Gailani *et al.*, *Nature Genet.* 14: 78-81 (1996); Hahn *et al.*, *Cell* 85: 841-51 (1996); Johnson *et al.*, *Science* 272: 1668-71 (1996); Unden *et al.*, *Cancer Res.* 56: 4562-5 (1996); Wicking *et al.*, *Am. J. Hum. Genet.* 60: 21-6 (1997). Loss of *Ptch* function is thought to cause an uncontrolled Smo signaling in basal cell carcinoma. Similarly, activating *Smo* mutations have been identified in sporatic BCC tumors (Xie *et al.*, *Nature* 391: 90-2 (1998)), emphasizing the role of *Smo* as the signaling subunit in the receptor complex for *SHh*. However, the exact mechanism by which *Ptch* controls *Smo* activity still has yet to be clarified and the signaling mechanisms by which the *Hh* signal is transmitted from the receptor to downstream targets also remain to be elucidated. Genetic epistatic analysis in Drosophila has identified several segment-polarity genes which appear to function as components of the *Hh* signal transduction pathway, Ingham, *Curr. Opin. Genet. Dev.* 5: 492-8 (1995); Perrimon, *supra*. These include a kinesin-like molecule, Costal-2 (*Cos-2*) [Robbins *et al.*, *Cell* 90: 225-34 (1997); Sisson *et al.*, *Cell* 90: 235-45 (1997)], a protein designated *fused* [Preat *et al.*, *Genetics* 135: 1047-62 (1993); Therond et al., *Proc. Natl Acad Sci. USA* 93: 4224-8 (1996)], a novel molecule with unknown function designated *Suppressor of fused* [Pham *et al.*, *Genetics* 140: 587-98 (1995); Preat. *Genetics* 132: 725-36 (1992)] and a zinc finger protein *Ci*. [Alexandre *et al.*, *Genes Dev.* 10: 2003-13 (1996); Dominguez *et al.*, *Science* 272: 1621-5 (1996); Orenic *et al*, *Genes Dev.* 4: 1053-67 (1990)]. Additional elements implicated in *Hh* signaling include the transcription factor CBP [*Akimaru et al.*, *Nature* 386: 735-738 (1997)], the negative regulator *slimb* [Jiang and Struhl, Nature 391: 493-496 (1998)] and the *SHh* response element COUP-TFII [Krishnan *et al.*, *Science* 278: 1947-1950 (1997)].

Mutants in *Cos-2* are embryonicly lethal and display a phenotype similar to *Hh* over expression, including duplications of the central component of each segment and expansion domain of *Hh* responsive genes. In contrast, mutant embryos for fused and Ci show a phenotype similar to *Hh* loss of function including deletion of the posterior part of each segment and replacement of a mirror-like image duplication of the anterior part or each segment and replacement of a mirror-like duplication of the anterior part, Busson *et al.*, *Roux. Arch. Dev. Biol.* 197: 221-230 (1988). Molecular characterizations of Ci suggested that it is a transcription factor which directly activates *Hh* responsive genes such as *Wingless* and *Dpp*, Alexandre *et al.*, (1996) *supra*, Dominguez *et al.*, (1996) *supra*. Likewise, molecular analysis of *fused* reveals that it is structurally related to serine threonine kinases and that both intact N-terminal kinase domain and a C-terminal regulatory region are required for its proper function, Preat *et al.*, *Nature* 347: 87-9 (1990); Robbins *et al.*, (1997), *supra*; Therond *et al.*, *Proc. Natl. Acad. Sci. USA* 93: 4224-8 (1996). Consistent with the putative opposing functions of Cos-2 and *fused*, *fused* mutations are suppressed by Cos-2 mutants and also by *Suppressor of fused* mutants, Preat *et al.*, *Genetics* 135: 1047-62 (1993). However, whereas *fused* null mutations and N-terminal kinase domain mutations can be fully suppressed by *Suppressor of fused* mutations, C-terminus mutations of fused display a strong Cos-2 phenotype in a *Suppressor of fused* background. This suggests that the fused kinase domain can act as a constitutive activator of *SHh* signaling

when *Suppressor of Fused* is not present. Recent studies have shown that the 92 kDa *Drosophila fused*. Cos-2 and Ci are present in a microtubule associated multiprotein complex and that Hh signaling leads to dissociation of this complex from microtubules, Robbins *et al*, *Cell* 90: 225-34 (1997); Sisson *et al*., *Cell* 90: 235-45 (1997). Both *fused* and Cos-2 become phosphorylated in response to *Hh* treatment, Robbins *et al*., *supra*; Therond *et al*., *Genetics* 142: 1181-98 (1996), but the kinase(s) responsible for this activity(ies) remain to be characterized. To date, the only known vertebrate homologues for these components are members of the *Gli* protein family (e.g., *Gli-1*, *Gli-2* and *Gli-3*). These are zinc finger putative transcription factors that are structurally related to *Ci*. Among these, *Gli*-1 was shown to be a candidate mediator of the *SHh* signal [Hynes *et al*., *Neuron* 15: 35-44 (1995), Lee *et al*., *Development* 124: 2537-52 (1997); Alexandre *et al*., *Genes Dev*. 10: 2003-13 (1996)] suggesting that the mechanism of gene activation in response to *Hh* may be conserved between fly and vertebrates. To determine whether other signaling components in the *Hh* cascade are evolutionarily conserved and to examine the function of fused in the *Hh* signaling cascade on the biochemical level, Applicants have isolated and characterized the human *fused* cDNA. Tissue distribution on the mouse indicates that fused is expressed in *SHh* responsive tissues. Biochemical studies demonstrate that fused is a functional kinase. Functional studies provide evidence that fused is an activator of *Gli* and that a dominant negative form of *fused* is capable of blocking *SHh* signaling in *Xenopus* embryos. Together this data demonstrated that *fused* is directly involved in *Hh* signaling.

Applicants have identified a cDNA encoding a human *fused* (h*fused*) polypeptide and thus have provided for the first time a vertebrate *fused* molecule.

SUMMARY OF THE INVENTION

In one embodiment, the invention provides an isolated nucleic acid molecule having at least about 80% sequence identity to (a) a DNA molecule encoding a fused polypeptide comprising the sequence of amino acids 1 to 260 of Fig. 1 (SEQ ID NO. 24), or (b) the complement of the DNA molecule of (a); and encoding a polypeptide having fused biological activity. The sequence identity preferably is about 85%, more preferably about 90%, most preferably about 95%. In one aspect, the isolated nucleic acid has at least about 80%, preferably at least about 85%, more preferably at least about 90%, and most preferably at least about 95% sequence identity with a polypeptide having amino acid residues 1 to about 1315 of Fig. 1 (SEQ ID NO. 2). Preferably, the highest degree of sequence identity occurs within the kinase domain (amino acids 1 to about 260 (SEQ ID NO:24 as shown in Fig. 1). Especially preferred are those nucleic acid molecule containing a coding sequence for a lysine at amino acid position 33. In a further aspect, the isolated nucleic acid molecule comprises DNA encoding a human *fused* polypeptide having amino acid residues 1 to about 260 (SEQ ID NO:24 as shown in Fig. 1). In yet another aspect, the invention provides for an isolated nucleic acid comprising DNA having at least a 95% sequence identity to (a) a DNA molecule encoding the same mature polypeptide encoded by the cDNA in ATCC Deposit No. 209637 (designation: pRK5tkneo.hFused-1272), alternatively the coding sequence of clone pRK5tkneo.hFused-1272, deposited under accession number ATCC 209637. In a still further aspect, the invention provides for a nucleic acid comprising human fused encoding sequence of the cDNA in ATCC deposit No. 209637 (designation: pRK5tkneo.hFused-1272) or a sequence which hybridizes thereto under stringent conditions.

In another embodiment, the invention provides a vector comprising DNA encoding a vertebrate

fused polypeptide. A host cell comprising such a vector is also provided. By way of example, the host cells may be mammalian cells, (e.g. CHO cells), prokaryotic cells (e.g., *E. coli*) or yeast cells (e.g., *Saccharomyces cerevisiae*). A process for producing vertebrate *fused* polypeptides is further provided and comprises culturing host cells under conditions suitable for expression of vertebrate *fused* and recovering the same from the cell culture.

In yet another embodiment, the invention provides an isolated vertebrate *fused* polypeptide. In particular, the invention provides isolated native sequence vertebrate *fused* polypeptide, which in one embodiment is a human fused including an amino acid sequence comprising residues 1 to about 1315 of Figure 1 (SEQ ID NO. 2). Human and other native vertebrate *fused* polypeptides with or without the initiating methionine are specifically included. Alternatively, the invention provides a vertebrate *fused* polypeptide encoded by the nucleic acid deposited under accession number ATCC 209637.

In yet another embodiment, the invention provides chimeric molecules comprising a vertebrate *fused* polypeptide fused to a heterologous polypeptide or amino acid sequence. An example of such a chimeric molecule comprises a vertebrate *fused* polypeptide fused to an epitope tag sequence or a constant region of an immunoglobulin.

In yet another embodiment, the invention provides an expressed sequence tag (EST) comprising the nucleotide sequences identified in Fig. 2 as 2515662 (SEQ ID NO. 3).

In yet another embodiment, the invention provides for compounds and methods for developing antagonists against and agonist promoting *fused* modulation of Hedgehog signaling. In particular, an antagonist of vertebrate fused which blocks, prevents, inhibits and/or neutralized the normal functioning of fused in *SH* signaling pathway, including both small bioorganic molecules and antisense nucleotides.

In yet another embodiment, the invention provides for alternatively spliced variants of human *fused*. In still yet a further embodiment, the invention provides a method of screening or assaying for identifying molecules that modulate the *fused* activation of hedgehog signaling. Preferably, the molecules either prevent interaction of *fused* with its associative complexing proteins or prevent or inhibit dissociation of complexes. The assay comprises the incubation of a mixture comprising fused and a substrate (*e.g., Gli, COUP-TFII, slimb, CBP, MBP*) with a candidate molecule and detection of the ability of the candidate molecule to modulate fused phosphorylation of its substrate. The screened molecules preferably are small molecule drug candidates. In particular, the method relates to a technique for screening for antagonists or agonists of *fused* biological activity comprising:

(a) exposing the fused expressing target cells in culture to a candidate compound; and
(b) analyzing cell lysates to asses the level and/or identity of phosphorylation; or
(c) scoring phenotypic or functional changes in treated cells;

and comparing the results to control cells which were not exposed to the candidate compound.

In yet another embodiment, the method relates to a technique of diagnosing to determine whether a particular disorder is modulated by hedgehog signaling, comprising:

(a) culturing test cells or tissues;
(b) administering a compound which can inhibit *fused* modulated *hedgehog* signaling; and
(c) measuring the degree of kinase attenuation on the *fused* substrate in cell lysates or

hedgehog mediated phenotypic effects in the test cells.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1E show the nucleotide (SEQ ID NO. 1) and derived amino acid sequence (SEQ ID NO. 2) of a native sequence of human fused polypeptide. Included are the kinase domain (residues 1 to about 260) (SEQ ID NO 24) and the ATP binding site at about amino acid position 33.

Figure 2 shows the EST 2515662 (SEQ ID NO. 3) that was used in the cloning of the human full-length sequence.

Figures 3A-3B show a comparison between human and *Drosophila* fused (SEQ ID NOS. 2 and 23, respectively). Gaps introduced for optimal alignment are indicated by dashes. Identical amino acids are boxed. The lysine residue mutated in fused-DN (dominant negative, lysine at amino acid position 33) is shown with a star.

Figures 4A-4E show the sequence of DNA28494 (SEQ ID NO. 6) that was an incorrectly spliced variant of human fused isolated from a fetal lung library. This clone contains a potential initiation methionine at position 116 followed by an open reading frame of 1944 bp. A second open reading frame is present from about position 2295 to 4349. There is one nucleotide difference between clone DNA28495 (SEQ ID NO. 4) and clone DNA28494 (SEQ ID NO. 6) located in the first ORF at position 1863 of clone 28495 (SEQ ID NO. 4) (A vs. G) which changes the coding sequence from an Gln to a Arg at position 583. The first open reading frame of DNA28494 (SEQ ID NO. 6) starts at residue 115 and is followed by a 630 amino acid long open reading frame.

Figures 5A-5E show shows sequence of DNA28494 (SEQ ID NO. 6) that was another incorrectly spliced variant of human fused isolated from a fetal lung library.

Figure 6 is a western blot of the PCR product of an epitope tag of DNA28495 (SEQ ID NOS. 5 & 21) and DNA28494 (SEQ ID NOS. 7 & 22). A specific band of 150 kDa was detected in the cell pellet of cells transfected with the construct corresponding to clone DNA28494 (SEQ ID NO. 6) and a specific band of approximately 100 kDa could be detected for clone DNA28495 (SEQ ID NO. 4) (Fig. 6). These bands were not present in the mock transfected control. The presence of the 100 kDa band suggests the two open reading frames of DNA28494 (SEQ ID NO. 6) can be spliced together to direct the synthesis of a large protein of 150 kDa. The absence of this band for DNA28495 (SEQ ID NO. 4) suggested that this clone apparently cannot be correctly spliced.

Figure 7 is a northern blot analysis of human *fused* (SEQ ID NO 1). Multiple human fetal and adult tissue northern blots were probes with a human *fused* cDNA probe.

Figures 8A-8F show is a photograph showing in situ hybridization of embryonic and adult tissues with *fused* (SEQ ID NO 1). Sagittal sections of E11.5 (Fig. 8A) and E13.5 (Fig. 8B) mouse embryos. Coronal section through the spinal chord of E11.5 (Fig. 8C) and E13.5 (Fig. 8D) mouse embryo. Sagittal section through P1 (Fig. 8E) and adult (Fig. 8F) mouse. Cp, choroid plexus; hb, hindbrain; hip, hippocampal formation; ht, heart; hy, hypothalamus; kd, kidney; lg, lung; mb, midbrain; md, midgut; mnd, mandibular component of first branchial arch; sc, spinal cord; st, stomach; tec, midbrain tectum; vh, ventral horn of spinal cord; vm, ventral midbrain. Scale bars: Fig. 8A, 1.0 mm; Fig. 8B, 1.62 mm; Fig. 8C, 0.14 mm; Fig. 8D, 0.17 mm; Fig. 8E, 2.0 mm; and Fig. 8F, 3.1 mm.

Figures 9A-9C are a photograph showing *in situ* hybridization showing the presence of *fused* mRNA in high levels in the adult mouse testes (Fig. 9A). High magnification reveals differences in levels of expression within seminiferous tubules (Fig. 9C). Hybridization of the testis with a sense strand control probe to *fused* gave no hybridization (Fig. 9B).

Figures 10A-10B are a bar graph representing the activation of Gli by *fused*. (Fig. 10A): C3H10T1/2 cells were cotransfected with a p9XGliLus, ptkRenilla luciferase and *fused* or various *fused* mutants. Cells were harvested 48h after transfection and the luciferase activity was assayed as described in Example 7. (Fig. 10B): *Fused* transactivation of a Gli reporter construct. C3H10T1/2 cells were cotransfected with a p9XGliLuc reporter construct, ptkRenilla luciferase and a CMV driven expression vector for fused or various fused mutants. Cells were harvested 48 hours after transfection and the luciferase activity was assayed as described in the Examples. The data represents the mean of duplicative determinations.

Figures 11A-11E are a photograph showing that *fused*-DN (SEQ ID NO 25) inhibits *SHh* signaling in early *Xenopus* development. Depicted are: (Fig. 11A) Dorsal view of tadpole stage embryos. Top embryo is *fused*-DN (SEQ ID NO 25) injection and bottom embryo is the control; (Fig. 11B) Side view of tadpole stage embryo. Top embryo is *fused*-DN (SEQ ID NO 25) injection and bottom embryo is the control; (Figs. 11C & 11D) Pax-6 staining of stage 16 neurula embryos injected with control DNA and *fused*-DN (SEQ ID NO 25), respectively; (Fig. 11E) *SHh* expression in the floor plate of neurula stage control embryo (left) or *fused*-DN (SEQ ID NO 25) injected embryo (right).

Figure 12 is a photograph which confirms the kinase activity of *fused* (SEQ ID NO 2) and its activation of Gli. Depicted are 293 cells transfected with HA tagged *fused* constructs as indicated in Example 10 and immunoprecipitated with anti-HA antibodies and protein A sepharose. Protein A beads were subjected to *in vitro* kinase assay as described in Example 10 in the presence of MBP.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

The terms "vertebrate *fused*" and "vertebrate *fused* polypeptide" when used herein encompass native sequence vertebrate *fused* and vertebrate *fused* variants (which are further defined herein) having *fused* biological activity. *Fused* may be isolated from a variety of sources, such as from human tissue types or from another source, or prepared by recombinant or synthetic methods.

A "native sequence vertebrate *fused*" comprises a polypeptide having the same amino acid sequence as a vertebrate *fused* derived from nature. Such native sequence vertebrate *fused* can be isolated from nature or can be produced by recombinant and/or synthetic means. The term "native sequence vertebrate *fused*" specifically encompasses naturally occurring truncated forms of vertebrate *fused*, naturally occurring variant forms (*e.g.*, alternatively spliced forms) and naturally-occurring allelic variants of vertebrate *fused*. Native vertebrate *fused* includes e.g., *fused* in mammals such as human, murine, bovine, porcine, equine, feline, canine, etc., and preferably refers to human. Thus, one embodiment of the invention, the native sequence human vertebrate *fused* is a mature or full-length native human vertebrate *fused* comprising amino acids 1 to 1315 of SEQ ID NO: 2 as shown in Fig. 1 with or without the initiating methionine at position 1.

"Vertebrate *fused* variant" means an active vertebrate *fused* as defined below having at least about

80% amino acid sequence identity to (a) a DNA molecule encoding a vertebrate *fused* polypeptide, or (b) the complement of the DNA molecule of (a). In a particular embodiment, the vertebrate *fused* variant has at least about 80% amino acid sequence homology with the vertebrate *fused* having the deduced amino acid sequence (SEQ ID NO:2) shown in Fig. 1 for a full-length native sequence vertebrate *fused*. Such vertebrate *fused* variants include, without limitation, vertebrate *fused* polypeptides wherein one or more amino acid residues are added, or deleted, at the N- or C-terminus of the sequence of Fig. 1 (SEQ ID NO 2). Preferably, the nucleic acid or amino acid sequence identity is at least about 85%, more preferably at least about 90%, and even more preferably at least about 95%.

"Percent (%) amino acid sequence identity" with respect to the vertebrate *fused* sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the vertebrate *fused* sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. "Percent (%) nucleic acid sequence identity" with respect to the vertebrate *fused* sequences identified herein is defined as the percentage of nucleotides in a candidate sequence that are identical with the nucleotides in the vertebrate *fused* sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared.

The term "epitope tagged" when used herein refers to a chimeric polypeptide comprising vertebrate *fused* polypeptide, or a portion thereof, fused to a "tag polypeptide". The tag polypeptide has enough residues to provide an epitope against which an antibody can be made, yet is short enough such that it does not interfere with activity of the vertebrate *fused* polypeptide. The tag polypeptide preferably also is fairly unique so that the antibody does not substantially cross-react with other epitopes. Suitable tag polypeptides generally have at least six amino acid residues and usually between about 8 to about 50 amino acid residues (preferably, between about 10 to about 20 residues).

As used herein, the term "immunoadhesin" designates antibody-like molecules which combine the binding specificity of a heterologous protein (an "adhesin") with the effector functions of immunoglobulin constant domains. Structurally, the immunoadhesin comprise a fusion of an amino acid sequence with the desired binding specificity which is other than the antigen recognition and binding site of an antibody (i.e., is "heterologous"), and an immunoglobulin constant domain sequence. The adhesin part of an immunoadhesin molecule typically is a contiguous amino acid sequence comprising at least the binding site of a receptor or a ligand. The immunoglobulin constant domain sequence in the immunoadhesins may be obtained from any

immunoglobulin, such as IgG-1, IgG-2, IgG-3 or IgG-4 subtypes, IgA (including IgA-1 and IgA-2, IgE, IgD or IgM. Immunoadhesion reported in the literature include fusions of the T cell receptor* [Gascoigne *et al.*, *Proc. Natl. Acad. Sci. USA* 84: 2936-2940 (1987)]; CD4* [Capron *et al.*, *Nature* 337: 525-531 (1989); Traunecker *et al.*, *Nature* 339: 68-70 (1989): Zettmeissl *et al.*, *DNA Cell Biol. USA* 9: 347-353 (1990); Byrn *et al.*, *Nature* 344, 667-670 (1990)]; L-selectin (homing receptor) [Watson *et al.*, *J. Cell. Biol.* 110, 2221-2229 (1990); Watson *et al.*, *Nature* 349, 164-167 (1991)]; CD44* [Aruffo *et al.*, *Cell* 61, 1303-1313 (1990)]; CD28* and B7* [Linsley *et al.*, *J. Exp. Med.* 173, 721-730 (1991)]; CTLA-4* [Lisley *et al.*, *J. Exp. Med.* 174, 561-569 (1991)]; CD22* [Stamenkovic *et al.*, *Cell* 66, 1133-1144 (1991)]; TNF receptor [Ashkenazi *et al.*, *Proc. Natl. Acad. Sci. USA* 88, 10535-10539 (1991); Lessiauer *et al.*, *Eur. J. Immunol.* 27, 2883-2886 (1991); Peppel *et al.*, *J. Exp. Med.* 174, 1483-1489 (1991)]; NP receptors [Bennett *et al.*, J. Biol. Chem. 266, 23060-23067 (1991)]; IgE receptor α-chain* [Ridgway and Gorman, J. Cell. Biol. 115, abstr. 1448 (1991)]; HGF receptor [Mark, M.R. *et al.*, 1992, submitted], where the asterisk (*) indicates that the receptor is member of the immunoglobulin superfamily.

"Stringency" of hybridization reactions is readily determinable by one of ordinary skill in the art, and generally is an empirical calculation dependent upon probe length, washing temperature, and salt concentration. In general, longer probes require higher temperatures for proper annealing, while shorter probes need lower temperatures. Hybridization generally depends upon the ability of denatured DNA to reanneal when complementary strands are present in an environment near but below their T^m (melting temperature). The higher the degree of desired homology between the probe and hybridizable sequence, the higher the relative temperature which can be used. As a result, it follows that higher relative temperatures would tend to make the reaction conditions more stringent, while lower temperatures less so. Moreover, stringency is also inversely proportional to salt concentrations. For additional details and explanation of stringency of hybridization reactions, see Ausubel *et al.*, *Current Protocols in Molecular Biology* (1995).

"Stringent conditions," as defined herein may be identified by those that: (1) employ low ionic strength and high temperature for washing, for example 0.015 M sodium chloride/0.0015 M sodium citrate/0.1% sodium dodecyl sulfate at 50°C; (2) employ during hybridization a denaturing agent, such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpurrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride, 75 mM sodium citrate at 42°C; (3) employ 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 μg/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC (sodium chloride/sodium citrate) and 50% formamide at 55°C, followed by a high-stringency wash consisting of 0.1 x SSC containing EDTA at 55°C.

"Moderately stringent conditions" may be identified as described by Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual* (New York: Cold Spring Harbor Laboratory Press, 1989), and include the use of a washing solution and hybridization conditions (e.g., temperature, ionic strength and %SDS) less stringent than described above. An example of moderately stringent conditions is a condition such as overnight incubation at 37°C in a solution comprising: 20% formamide, 5 x SSC (150 mM NaCl, 15 mM

trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5 x Denhardt's solution, 10% dextran sulfate, and 20 mg/mL denatured sheared salmon sperm DNA, followed by washing the filters in 1 x SSC at about 37-50°C. The skilled artisan will recognize how to adjust the temperature, ionic strength, *etc.* as necessary to accommodate factors such as probe length and the like.

"Isolated," when used to describe the various polypeptides disclosed herein, means polypeptide that has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the polypeptide will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated polypeptide includes polypeptide *in situ* within recombinant cells, since at least one component of the vertebrate *fused* natural environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at least one purification step.

An "isolated" vertebrate *fused* nucleic acid molecule is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the vertebrate *fused* nucleic acid. An isolated vertebrate *fused* nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated vertebrate *fused* nucleic acid molecules therefore are distinguished from the corresponding native vertebrate *fused* nucleic acid molecule as it exists in natural cells.

The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

The term "antibody" is used in the broadest sense and specifically covers single monoclonal antibodies (including agonist and antagonist antibodies), antibody compositions with polyepitopic specificity, as well as antibody fragments (e.g., Fab, $F(ab')_2$ and Fv), so long as they exhibit the desired biological activity.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical

except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. In addition to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler & Milstein, Nature 256:495 (1975), or may be made by recombinant DNA methods [see, e.g. U.S. Patent No. 4,816,567 (Cabilly *et al.*)].

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity [U.S. Patent No. 4,816,567; Cabilly *et al.*; Morrison *et al.*, Proc. Natl. Acad. Sci. USA 81, 6851-6855 (1984)].

"Humanized" forms of non-human (e.g. murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, corresponding non-human residues replace Fv framework residues of the human immunoglobulin. Furthermore, humanized antibody may comprise residues that are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details see: Jones *et al.*, Nature 321, 522-525 (1986); Reichmann *et al.*, Nature 332, 323-329 (1988); Presta, Curr. Op. Struct. Biol. 2 593-596 (1992) and U.S. Patent No. 5,225,539 (Winter) issued July 6, 1993.

"Active" or "activity" for the purposes herein refers to form(s) of vertebrate *fused* which retain the biologic and/or immunologic activities of native or naturally occurring vertebrate *fused*. A preferred activity is the ability to bind to and affect, e.g., block or otherwise modulate, hedgehog signaling. The activity preferably involves the regulation of the pathogenesis of Basal cell carcinoma. Another preferred biological

activity is the ability to phosphorylate or modulate the phosphorylation of Gli.

The term "antagonist" is used herein in the broadest sense to include any molecule which blocks, prevents, inhibits, neutralizes the normal functioning of *fused* in the *Hh* signaling pathway. One particular form of antagonist includes a molecule that interferes with the interaction between fused and its binding or complexing proteins. In a similar manner, the term "agonist" is used herein to include any molecule which promotes, enhances or stimulates the normal functioning of *fused* in the *Hh* signaling pathway. Suitable molecules that affect the protein-protein interaction of *fused* and its binding proteins include fragments of the latter or small bioorganic molecules, e.g., peptidomimetics, which will prevent or enhance, as the case may be, the interaction of proper complex formation. Non-limiting examples include proteins, peptides, glycoproteins, glycopeptides, glycolipids, polysaccharides, oligosaccharides, nucleic acids, bioorganic molecules, peptidomimetics, pharmacological agents and their metabolites, transcriptional and translation control sequences, and the like. Another preferred form of antagonist includes antisense nucleotides that inhibit proper transcription of wild type *fused*. Preferred forms of antagonists and are small molecules, which specifically bind to or block binding of the ATP binding site of *fused*.

The term "modulation" or "modulating" means upregulation or downregulation of a signaling pathway. Cellular processes under the control of signal transduction may include, but are not limited to, transcription of specific genes; normal cellular functions, such as metabolism, proliferation, differentiation, adhesion, apoptosis and survival, as well as abnormal processes, such as transformation, blocking of differentiation and metastasis.

The techniques of "polymerase chain reaction," or "PCR", as used herein generally refers to a procedure wherein minute amounts of a specific piece of nucleic acid, RNA and/or DNA are amplified as described in U.S. Pat. No. 4,683,195 issued 28 July 1987. Generally, sequence information from the ends of the region of interest or beyond needs to be available, such that oligonucleotide primers can be designed: these primer will be identical or similar in sequence to opposite strands of the template to be amplified. The 5' terminal nucleotides of the two primers may coincide with the ends of the amplified material. PCR sequences form total genomic DNA, and cDNA transcribed from total cellular RNA, bacteriophage, or plasmid sequences, etc. See generally Mullis *et al., Cold Spring Harbor Symp. Quant. Biol.* 51: 263 (1987); Erlich, Ed., PCR Technology, (Stockton Press, NY, 1989). As used herein, PCR is considered to be one, but not the only, example of a nucleic acid test sample comprising the use of a known nucleic acid as a primer and a nucleic acid polymerase to amplify or generate a specific piece of nucleic acid.

II. Compositions and Methods of the Invention

A. Full-length vertebrate *fused*

The present invention provides newly identified and isolated nucleotide sequences encoding polypeptides referred to in the present application as human and vertebrate *fused*. In particular, Applicants have identified and isolated cDNA encoding a vertebrate *fused* polypeptide, as disclosed in further detail in the Examples below. Using BLAST, BLAST-2 and FastA sequence alignment computer programs, Applicants found that a full-length native sequence human *fused* (shown in Figure 3 (SEQ ID NO 2)) has 28% amino acid sequence identity with *Drosophila fused* (SEQ ID NO 23). Accordingly, it is presently believed that the human *fused* disclosed in the present application is a newly identified member of the

hedgehog signaling cascade.

The full-length native sequence of human vertebrate *fused* gene, or portions thereof, may be used as hybridization probes for a cDNA library to isolate the full-length gene or to isolate still other vertebrate homolog genes (for instance, those encoding naturally-occurring variants of vertebrate *fused* or vertebrate *fused* from other species) which have a desired sequence identity to the vertebrate *fused* sequence disclosed in Fig.1 (SEQ ID NO 1). Optionally, the length of the probes will be about 20 to about 50 bases. The hybridization probes may be derived from the nucleotide sequence of Fig. 1 (SEQ ID NO 1) or from genomic sequences including promoters, enhancer elements and introns of native sequence vertebrate *fused*. By way of example, a screening method will comprise isolating the coding region of the vertebrate *fused* gene using the known DNA sequence to synthesize a selected probe of about 40 bases. Hybridization probes may be labeled by a variety of labels, including radionucleotides such as ^{32}P or ^{35}S, or enzymatic labels such as alkaline phosphatase coupled to the probe via avidin/biotin coupling systems. Labeled probes having a sequence complementary to that of the vertebrate *fused* gene of the present invention can be used to screen libraries of human cDNA, genomic DNA or mRNA to determine which members of such libraries the probe hybridizes to.

B. Vertebrate *fused* Variants

In addition to the full-length native sequence vertebrate *fused* described herein, it is contemplated that vertebrate *fused* variants can be prepared. Vertebrate *fused* variants can be prepared by introducing appropriate nucleotide changes into a known vertebrate *fused* DNA, or by synthesis of the desired vertebrate *fused* polypeptides. Those skilled in the art will appreciate that amino acid changes may alter post-translational processes of the vertebrate *fused*.

Variations in the native full-length sequence vertebrate *fused* or in various domains of the vertebrate *fused* described herein, can be made, for example, using any of the techniques and guidelines for conservative and non-conservative mutations set forth, for instance, in U.S. Patent No. 5,364,934. Variations may be a substitution, deletion or insertion of one or more codons encoding the vertebrate *fused* that results in a change in the amino acid sequence of the vertebrate *fused* as compared with the native sequence vertebrate *fused*. Optionally the variation is by substitution of at least one amino acid with any other amino acid in one or more of the domains of the vertebrate *fused*. Guidance in determining which amino acid residue may be inserted, substituted or deleted without adversely affecting the desired activity may be found by comparing the sequence of the vertebrate *fused* with that of homologous known protein molecules and minimizing the number of amino acid sequence changes made in regions of high homology. Amino acid substitutions can be the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, such as the replacement of a leucine with a serine, i.e., conservative amino acid replacements. Insertions or deletions may optionally be in the range of 1 to 5 amino acids. The variation allowed may be determined by systematically making insertions, deletions or substitutions of amino acids in the sequence and testing the resulting variants for activity in the *in vitro* assay described in the Examples below.

The variations can be made using methods known in the art such as oligonucleotide-mediated (site-

directed) mutagenesis, alanine scanning, and PCR mutagenesis. Site-directed mutagenesis [Carter et al., Nucl. Acids Res., 13:4331 (1986); Zoller et al., Nucl. Acids Res., 10:6487 (1987)], cassette mutagenesis [Wells et al., Gene, 34:315 (1985)], restriction selection mutagenesis [Wells et al., Philos. Trans. R. Soc. London SerA, 317:415 (1986)] or other known techniques can be performed on the cloned DNA to produce the vertebrate *fused* variant DNA.

Scanning amino acid analysis can also be employed to identify one or more amino acids along a contiguous sequence. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine, and cysteine. Alanine is typically a preferred scanning amino acid among this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant. Alanine is also typically preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions [Creighton, The Proteins, (W.H. Freeman & Co., N.Y.); Chothia, *J. Mol. Biol.*, 150:1 (1976)]. If alanine substitution does not yield adequate amounts of variant, an isoteric amino acid can be used.

In the human fused sequence depicted in Figure 1, the kinase domain is represented by amino acid residues 1-260 (SEQ ID NO 24) of which position lysine 33 appears to be necessary for ATP binding and thus enzymatic activity.

C. Modifications of vertebrate *fused*

Covalent modifications of vertebrate *fused* are included within the scope of this invention. One type of covalent modification includes reacting targeted amino acid residues of the vertebrate *fused* with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C- terminal residues of the vertebrate *fused*. Derivatization with bifunctional agents is useful, for instance, for crosslinking vertebrate *fused* to a water-insoluble support matrix or surface for use in the method for purifying anti-vertebrate *fused* antibodies, and vice-versa. Commonly used crosslinking agents include, *e.g.*, 1,1-bis(diazo-acetyl)-2-phenylethane, glutaraldehyde, N-hydroxy-succinimide esters, for example, esters with 4-azido-salicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis-(succinimidyl-propionate), bifunctional maleimides such as bis-N-maleimido-1,8-octane and agents such as methyl-3-[(p-azidophenyl)-dithio]pro-pioimi-date.

Other modifications include deamidation of glutaminyl and asparaginyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the α-amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)], acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification of vertebrate *fused* comprises linking the vertebrate *fused* polypeptide to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337. Such modifications would be expected in increase the half-life of the molecules in circulation in a mammalian system; Extended half-life of *fused* molecules might be useful under certain circumstances, such as where the fused variant is administered as a therapeutic agent.

The vertebrate *fused* of the present invention may also be modified in a way to form a chimeric molecule comprising vertebrate *fused* bonded to another, heterologous polypeptide or amino acid sequence. In one embodiment, such a chimeric molecule comprises a fusion of the vertebrate *fused* with a tag polypeptide, which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino- or carboxyl- terminus of the vertebrate *fused*. The presence of such epitope-tagged forms of the vertebrate *fused* can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the vertebrate *fused* to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag. In an alternative embodiment, the chimeric molecule may comprise a fusion of the vertebrate *fused* with an immunoglobulin or a particular region of an immunoglobulin. For a bivalent form of the chimeric molecule, such a fusion could be to the Fc region of an IgG molecule.

Ordinarily, the C-terminus of a contiguous amino acid sequence of a ligand- (IFN-γ-) binding domain of an IFN-γ receptor is fused to the N-terminus of a contiguous amino acid sequence of an immunoglobulin constant region, in place of the variable region(s), however N-terminal fusions are also possible.

Typically, such fusions retain at least functionally active hinge, CH2 and CH3 domains of the constant region of an immunoglobulin heavy chain. Fusions are also made to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the CH1 of the heavy chain or the corresponding region of the light chain. This ordinarily is accomplished by constructing the appropriate DNA sequence and expressing it in recombinant cell culture. Alternatively, immunoadhesins may be synthesized according to known methods.

The precise site at which the fusion is made is not critical; particular sites are well known and may be selected in order to optimize the biological activity, secretion or binding characteristics of the immunoadhesins.

In a preferred embodiment, the C-terminus of a contiguous amino acid sequence which comprises the binding site(s) for IFN-γ is fused, at the N-terminal end, to the C-terminal portion of an antibody (in particular the Fc domain), containing the effector functions of an immunoglobulin, e.g. immunoglobulin G_1 (IgG-1). As hereinabove mentioned, it is possible to fuse the entire heavy chain constant region to the sequence containing the binding site(s). However, more preferably, a sequence beginning in the hinge region just upstream of the papain cleavage site (which defines IgG Fc chemically; residue 216, taking the first residue of heavy chain constant region to be 114 [Kobet *et al.*, supra], or analogous sites of other immunoglobulins) is used in the fusion. Although it was earlier thought that in immunoadhesins the immunoglobulin light chain would be required for efficient secretion of the heterologous protein-heavy chain fusion proteins, it has been found that even the immunoadhesins containing the whole IgG1 heavy chain are efficiently secreted in the absence of light chain. Since the light chain is unnecessary, the immunoglobulin heavy chain constant domain sequence used in the construction of the immunoadhesins of the present invention may be devoid of a light chain binding site. This can be achieved by removing or sufficiently altering immunoglobulin heavy chain sequence elements to which the light chain is ordinarily linked so that such binding is no longer possible. Thus, the CH1 domain can be entirely removed in certain embodiments of the IFN-γ receptor-immunoglobulin chimeras.

In a particularly preferred embodiment, the amino acid sequence containing the extracellular domain of an IFN-γ receptor is fused to the hinge region and CH2, CH3; or CH1, hinge, CH2 and CH3 domains of an IgG-1, IgG-2, IgG-3, or IgG-4 heavy chain. The construction of a typical structure is disclosed in Example 1.

In some embodiments, the IFN-γ receptor-immunoglobulin molecules (immunoadhesins) are assembled as monomers, dimers or multimers, and particularly as dimers or tetramers. Generally, these assembled immunoadhesins will have known unit structures similar to those of the corresponding immunoglobulins. A basic four chain structural unit (a dimer of two immunoglobulin heavy chain-light chain pairs) is the form in which IgG, IgA and IgE exist. A four chain unit is repeated in the high molecular weight immunoglobulins; IgM generally exists as a pentamer of basic four-chain units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in a multimeric form in serum. In the case of multimers, each four chain unit may be the same or different.

It is not necessary that the entire immunoglobulin portion of the IFN-γ receptor-immunoglobulin chimeras be from the same immunoglobulin. Various portions of different immunoglobulins may be combined, and variants and derivatives of native immunoglobulins can be made as hereinabove described with respect to IFN-γ, in order to optimize the properties of the immunoadhesin molecules. For example, immunoadhesin constructs in which the hinge of IgG-1 was replaced with that of IgG-3 were found to be functional and showed pharmacokinetics comparable to those of immunoadhesins comprising the entire IgG-1 heavy chain.

Various tag polypeptides and their respective antibodies are well known in the art. Examples include poly-histidine (poly-his) or poly-histidine-glycine (poly-his-gly) tags; the flu HA tag polypeptide and its antibody 12CA5 [Field et al., Mol. Cell. Biol., 8:2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky et al., Protein Engineering, 3(6):547-553 (1990)]. Other tag polypeptides include the Flag-peptide [Hopp et al., BioTechnology, 6:1204-1210 (1988)]; the KT3 epitope peptide [Martin et al., Science, 255:192-194 (1992)]; an α-tubulin epitope peptide [Skinner et al., J. Biol. Chem., 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide tag [Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397 (1990)]. A preferred tag is the influenza HA tag.

D. Preparation of vertebrate *fused*

The description below relates primarily to production of a particular vertebrate *fused* by culturing cells transformed or transfected with a vector containing vertebrate *fused* nucleic acid. It is, of course, contemplated that alternative methods, which are well known in the art, may be employed to prepare vertebrate *fused*. For instance, the vertebrate *fused* sequence, or portions thereof, may be produced by direct peptide synthesis using solid-phase techniques [see, *e.g.*, Stewart et al., Solid-Phase Peptide Synthesis, W.H. Freeman Co., San Francisco, CA (1969); Merrifield, J. Am. Chem. Soc., 85:2149-2154 (1963)]. *In vitro* protein synthesis may be performed using manual techniques or by automation. Automated synthesis may be accomplished, for instance, using an Applied Biosystems Peptide Synthesizer (Foster City, CA) using manufacturer's instructions. Various portions of the vertebrate *fused* may be chemically synthesized separately and combined using chemical or enzymatic methods to produce the full-length vertebrate *fused*.

1. Isolation of DNA Encoding vertebrate *fused*

DNA encoding vertebrate *fused* may be obtained from a cDNA library prepared from tissue believed to possess the vertebrate *fused* mRNA and to express it at a detectable level. Accordingly, human vertebrate *fused* DNA can be conveniently obtained from a cDNA library prepared from human tissue, such

as described in the Examples. The vertebrate *fused*-encoding gene may also be obtained from a genomic library or by oligonucleotide synthesis.

Libraries can be screened with probes (such as antibodies to the vertebrate *fused* or oligonucleotides of at least about 20-80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures, such as described in Sambrook et al., Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989). An alternative means to isolate the gene encoding vertebrate *fused* is to use PCR methodology [Sambrook et al., supra; Dieffenbach et al., PCR Primer: A Laboratory Manual (Cold Spring Harbor Laboratory Press, 1995)].

The Examples below describe techniques for screening a cDNA library. The oligonucleotide sequences selected as probes should be of sufficient length and sufficiently unambiguous that false positives are minimized. The oligonucleotide is preferably labeled such that it can be detected upon hybridization to DNA in the library being screened. Methods of labeling are well known in the art, and include the use of radiolabels like ^{32}P-labeled ATP, biotinylation or enzyme labeling. Hybridization conditions, including moderate stringency and high stringency, are provided in Sambrook et al., supra.

Sequences identified in such library screening methods can be compared and aligned to other known sequences deposited and available in public databases such as GenBank or other private sequence databases. Sequence identity (at either the amino acid or nucleotide level) within defined regions of the molecule or across the full-length sequence can be determined through sequence alignment using computer software programs such as BLAST, BLAST-2, ALIGN, DNAstar, and INHERIT which employ various algorithms to measure homology.

Nucleic acid having protein coding sequence may be obtained by screening selected cDNA or genomic libraries using the deduced amino acid sequence disclosed herein for the first time, and, if necessary, using conventional primer extension procedures as described in Sambrook et al., supra, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

2. Selection and Transformation of Host Cells

Host cells are transfected or transformed with expression or cloning vectors described herein for vertebrate *fused* production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. The culture conditions, such as media, temperature, pH and the like, can be selected by the skilled artisan without undue experimentation. In general, principles, protocols, and practical techniques for maximizing the productivity of cell cultures can be found in *Mammalian Cell Biotechnology: A Practical Approach*, M. Butler, ed. (IRL Press, 1991) and Sambrook *et al., supra*.

Methods of transfection are known to the ordinarily skilled artisan, for example, $CaPO_4$ and electroporation. Depending on the host cell used, transformation is performed using standard techniques appropriate to such cells. The calcium treatment employing calcium chloride, as described in Sambrook et al., supra, or electroporation is generally used for prokaryotes or other cells that contain substantial cell-wall barriers. Infection with *Agrobacterium tumefaciens* is used for transformation of certain plant cells, as described by Shaw et al., *Gene*, 23:315 (1983) and WO 89/05859 published 29 June 1989. For mammalian

cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, *Virology* 52:456-457 (1978) can be employed. General aspects of mammalian cell host system transformations have been described in U.S. Patent No. 4,399,216. Transformations into yeast are typically carried out according to the method of Van Solingen *et al., J. Bact.*, 130:946 (1977) and Hsiao *et al., Proc. Natl. Acad. Sci. (USA)*, 76:3829 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, *e.g.*, polybrene, polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown *et al., Methods in Enzymology*, 185:527-537 (1990) and Mansour *et al., Nature*, 336:348-352 (1988).

Suitable host cells for cloning or expressing the DNA in the vectors herein include prokaryote, yeast, or higher eukaryote cells. Suitable prokaryotes include but are not limited to eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriae such as *E. coli*. Various *E. coli* strains are publicly available, such as *E. coli* K12 strain MM294 (ATCC 31,446); *E. coli* X1776 (ATCC 31,537); *E. coli* strain W3110 (ATCC 27,325) and K5 772 (ATCC 53,635).

In addition to prokaryotes, eukaryotic microbes such as filamentous fungi or yeast are suitable cloning or expression hosts for vertebrate *fused*-encoding vectors. *Saccharomyces cerevisiae* is a commonly used lower eukaryotic host microorganism.

Suitable host cells for the expression of vertebrate *fused* are derived from multicellular organisms. Examples of invertebrate cells include insect cells such as Drosophila S2 and Spodoptera Sf9, as well as plant cells. Examples of useful mammalian host cell lines include Chinese hamster ovary (CHO) and COS cells. More specific examples include monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture, Graham *et al., J. Gen Virol.*, 36:59 (1977)); Chinese hamster ovary cells/-DHFR (CHO, Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA*, 77:4216 (1980)); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); and mouse mammary tumor (MMT 060562, ATCC CCL51). The selection of the appropriate host cell is deemed to be within the skill in the art.

3. Selection and Use of a Replicable Vector

The nucleic acid (*e.g.*, cDNA or genomic DNA) encoding vertebrate *fused* may be inserted into a replicable vector for cloning (amplification of the DNA) or for expression. Various vectors are publicly available. The vector may, for example, be in the form of a plasmid, cosmid, viral particle, or phage. The appropriate nucleic acid sequence may be inserted into the vector by a variety of procedures. In general, DNA is inserted into an appropriate restriction endonuclease site(s) using techniques known in the art. Vector components generally include, but are not limited to, one or more of a signal sequence, an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. Construction of suitable vectors containing one or more of these components employs standard ligation techniques, which are known to the skilled artisan.

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria,

the 2: plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells. A preferred replicable expression vector is the plasmid is pRK5. *Holmes et al., Science*, 253:1278-1280 (1991).

Expression and cloning vectors will typically contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxins, *e.g.*, ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, *e.g.*, the gene encoding D-alanine racemase for *Bacilli*.

An example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the vertebrate *fused* nucleic acid, such as DHFR or thymidine kinase. An appropriate host cell when wild-type DHFR is employed is the CHO cell line deficient in DHFR activity, prepared and propagated as described by Urlaub *et al., Proc. Natl. Acad. Sci. USA*, 77:4216 (1980). A suitable selection gene for use in yeast is the *trp*1 gene present in the yeast plasmid YRp7 [Stinchcomb *et al., Nature*, 282:39 (1979); Kingsman *et al., Gene*, 7:141 (1979); Tschemper *et al., Gene*, 10:157 (1980)]. The *trp*1 gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEP4-1 [Jones, *Genetics*, 85:12 (1977)].

Expression and cloning vectors usually contain a promoter operably linked to the vertebrate *fused* nucleic acid sequence to direct mRNA synthesis. Promoters recognized by a variety of potential host cells are well known. Promoters suitable for use with prokaryotic hosts include the β-lactamase and lactose promoter systems [Chang *et al., Nature*, 275:615 (1978); Goeddel *et al., Nature*, 281:544 (1979)], alkaline phosphatase, a tryptophan (trp) promoter system [Goeddel, *Nucleic Acids Res.*, 8:4057 (1980); EP 36,776], and hybrid promoters such as the tac promoter [deBoer *et al., Proc. Natl. Acad. Sci. USA*, 80:21-25 (1983)]. Promoters for use in bacterial systems also will contain a Shine-Dalgarno (S.D.) sequence operably linked to the DNA encoding vertebrate *fused*.

Examples of suitable promoting sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase [Hitzeman *et al., J. Biol. Chem.*, 255:2073 (1980)] or other glycolytic enzymes [Hess *et al., J. Adv. Enzyme Reg.*, 7:149 (1968); Holland, *Biochemistry*, 17:4900 (1978)], such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phospho-fructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase.

Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657.

Vertebrate *fused* transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 July 1989), adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and Simian Virus 40 (SV40), from heterologous mammalian

promoters, *e.g.*, the actin promoter or an immunoglobulin promoter, and from heat-shock promoters, provided such promoters are compatible with the host cell systems.

Inserting an enhancer sequence into the vector may increase transcription of a DNA encoding the vertebrate fused by higher eukaryotes. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, α-fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. The enhancer may be spliced into the vector at a position 5' or 3' to the vertebrate *fused* coding sequence, but is preferably located at a site 5' from the promoter.

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and, occasionally 3', untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding vertebrate *fused*.

Still other methods, vectors, and host cells suitable for adaptation to the synthesis of vertebrate *fused* in recombinant vertebrate cell culture are described in Gething *et al., Nature*, 293:620-625 (1981); Mantei *et al., Nature*, 281:40-46 (1979); EP 117,060; and EP 117,058.

4. Detecting Gene Amplification/Expression

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA, 77:5201-5205 (1980)], dot blotting (DNA analysis), or *in situ* hybridization, using an appropriately labeled probe, based on the sequences provided herein. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal, and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native sequence vertebrate *fused* polypeptide or against a synthetic peptide based on the DNA sequences provided herein or against exogenous sequence fused to vertebrate *fused* DNA and encoding a specific antibody epitope.

5. Purification of Polypeptide

Forms of vertebrate *fused* may be recovered from host cell lysates. If membrane-bound, it can be released from the membrane using a suitable detergent solution (*e.g.* Triton-X 100) or by enzymatic

cleavage. Cells employed in expression of vertebrate *fused* can be disrupted by various physical or chemical means, such as freeze-thaw cycling, sonication, mechanical disruption, or cell lysing agents.

It may be desired to purify vertebrate *fused* from recombinant cell proteins or polypeptides. The following procedures are exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; protein A Sepharose columns to remove contaminants such as IgG; and metal chelating columns to bind epitope-tagged forms of the vertebrate *fused*. Various methods of protein purification may be employed and such methods are known in the art and described for example in Deutscher, Methods in Enzymology, 182 (1990); Scopes, Protein Purification: Principles and Practice, Springer-Verlag, New York (1982). The purification step(s) selected will depend, for example, on the nature of the production process used and the particular vertebrate *fused* produced.

E. Uses for vertebrate *fused*

(1) Fused is universal mediator of Hh signaling

The human *fused* full length molecule of (Fig. 1 (SEQ ID NO 1)) encodes a protein with a predicted molecular weight of 150 kDa, which is significantly larger that *Drosophila fused* (100 kDa, d*fused* (SEQ ID NO 23)). Human *fused* (h*fused*) shows notable homology to the Drosophila homologue in the kinase domain, but little homology with d*fused* or any other known protein over the remaining ≈1000 amino acids. The kinase domain extends from residue 1 to about residue 260, as is represented in Fig. 1 (SEQ ID NOS. 24 & 2). This divergence at the C-terminus of the molecules is unexpected given that the C-terminus of the *Drosophila* molecule is required for its activity, Preat *et al., Nature* 347: 87-9 (1990). An ATP binding site is at about amino acid position 33 and is required for kinase activity.

Prior studies in *Drosophila* indicate that d*fused* is necessary for *Hh* signal to occur but have not addressed the issue whether *fused* is sufficient to activate this signaling system. As depicted in the Examples, applicants have herein used a *Gli* DNA binding element present in the HNF3β promoter, in front of a luciferase mediator of the *Hh* cascade, which clearly demonstrates that *fused* alone is capable of activating *Gli* mediated transcription in this system. It is further apparent that both an intact kinase domain and an intact C-terminal non-catalytic domain are required for this activation, which supports the notion that *fused* functions as a kinase and that the C-terminus may play a role in the substrate recognition or in regulating the kinase activity.

Applicants have shown in the present application that h*fused* is a kinase which is capable of phosphorylating artificial substrates such as MBP. However, the identity of the physiological substrate for h*fused* remains to be determined. One obvious candidate is *Gli*-1 itself, as *Gli*-1 phosphorylation by h*fused* can be detected *in vitro*.

To determine if human *fused* is essential for *Hh* signaling in vertebrates, a mutant was constructed by altering a conserved lysine in the ATP binding site (about amino acid residue 33). Typically, such mutants act as inhibitor of the corresponding wild type kinase by blocking access to substrate and/or regulatory factors, He *et al., Nature* 374, 617-22 (1995). When overexpressed in 2-cell stage *Xenopus* embryos, the most remarkable phenotype was the presence of fused eyes in about 30% of the injected

embryos. Several lines of evidence indicate that this phenotype is likely to result from the inhibition of *Hh* signaling. First, *SHh* knockouts display a cyclopia phenotype attributed recently to mutations in the *SHh* gene. Chiang *et al., Nature* 383: 407-13 (1996). Second, zebrafish embryos (cyclops) with reduced expression of *SHh* or injected with constitutively active form of PKA, a negative regulator of the *Hh* pathway are cyclops. Third, *SHh*, emanating from prechordal plate, has been shown to inhibit expression of Pax-6, a key transcription factor required for eye development, in the center of a continuous eyefield, Ekker *et al., Curr. Biol.* 5: 944-55 (1995); Li *et al., Development* 124: 603-15 (1997); Macdonald *et al., Development* 121: 3267-78 (1995). Finally, staining for Pax-6 embryos injected with fused-DN revealed a single field of expression suggesting a failure of *SHh* emanating from the prechordal plate to downregulate the expression of Pax-6 at the center of the eyefield.

To confirm the position of *fused* in the *Hh* signaling pathway, expression of *SHh* in the floor plate of *Xenopus* embryos injected with h*fused*-DN could be rescued by coinjection of *Gli*-1. This suggests that *fused* acts in association with *Gli* in the *SHh* signaling pathway.

The tissue distribution of *fused* shows that it is expressed in all *SHh* responsive cells. In particular, its expression pattern overlaps well with *Ptch*, the binding component of the *Hh* receptor which is itself a target gene of the *SHh* signaling pathway. These data suggest that *fused* is involved in mediating a wide variety of effect *SHh* has on different tissues. Functionally, this was observed again in frog embryos where, *fused*-DN inhibited eye development as well as *SHh* expression in the floor plate.

h*Fused*-DN also appears to affect normal development of tissues such as the frog gut which is regulated by Indian *Hh*. This, combined with the fact that *fused* is expressed in the gut and testis, sites of *IHh* and *DHh* action respectively, suggest that *fused* may be a universal mediator of signaling for all members of the *Hh* protein family.

Very high levels of *fused* mRNA was found on germ cell, the development of which appears to be regulated by *DHh*. Homozygous mutant mice for *DHh* fail to develop germ cells and are viable but sterile (Bitgood *et al., Curr. Biol.* 6: 298-304 (1996). However, *Patched*, a Hedgehog receptor is expressed on interstitial Leydig cells and not on germ cells where *fused* is expressed, Bitgood *et al, supra*. This discrepancy suggests that there may be additional hedgehog receptors.

Applicants have shown in the Examples that wild type h*fused* is capable of activating *Gli* in a reporter assay. Furthermore, expression of *SHh* in the floor plate of frog embryos injected with h*fused*-DN could be rescued by coinjection of *Gli*-1. Taken together these observations are consistent with the assertion that *fused* acts downstream of *Smo* and upstream of *Gli* in this signaling pathway, which is consistent with the genetic evidence in *Drosophila* to date.

(2) General uses for vertebrate fused

Nucleotide sequences (or their complement) encoding vertebrate *fused* have various applications in the art of molecular biology, including uses as hybridization probes, in chromosome and gene mapping and in the generation of anti-sense RNA and DNA. Vertebrate *fused* nucleic acid will also be useful for the preparation of vertebrate *fused* polypeptides by the recombinant techniques described herein.

The full-length native sequence vertebrate *fused* gene, or portions thereof, may be used as hybridization probes for a cDNA library to isolate the full-length gene or to isolate still other genes (for

instance, those encoding naturally-occurring variants of vertebrate *fused* or vertebrate *fused* from other species) which have a desired sequence identity to the vertebrate *fused* sequence disclosed in Fig.1 (SEQ ID NO 1). Optionally, the length of the probes will be about 20 to about 50 bases. The hybridization probes may be derived from the nucleotide sequence of Fig. 1 (SEQ ID NO 1) or from genomic sequences including promoters, enhancer elements and introns of native sequence vertebrate *fused*. By way of example, a screening method will comprise isolating the coding region of the vertebrate *fused* gene using the known DNA sequence to synthesize a selected probe of about 40 bases. Hybridization probes may be labeled by a variety of labels, including radionucleotides such as ^{32}P or ^{35}S, or enzymatic labels such as alkaline phosphatase coupled to the probe via avidin/biotin coupling systems. Labeled probes having a sequence complementary to that of the vertebrate *fused* gene of the present invention can be used to screen libraries of human cDNA, genomic DNA or mRNA to determine to which members of such libraries the probe hybridizes. Hybridization techniques are described in further detail in the Examples below.

The probes may also be employed in PCR techniques to generate a pool of sequences for identification of closely related vertebrate *fused* sequences.

Nucleotide sequences encoding a vertebrate *fused* can also be used to construct hybridization probes for mapping the gene, which encodes vertebrate *fused* and for the genetic analysis of individuals with genetic disorders. The nucleotide sequences provided herein may be mapped to a chromosome and specific regions of a chromosome using known techniques, sucn as *in situ* hybridization, linkage analysis against known chromosomal markers, and hybridization screening with libraries.

Vertebrate *fused* polypeptides can be used in assays to identify the other proteins or molecules involved in complexing with fused which ultimately results in the modulation of hedgehog signaling. Alternatively, these molecules can modulate the fused kinase phosphorylation of its substrate. By such methods, inhibitors of the binding interaction can be identified. Proteins involved in such binding interactions can also be used to screen for peptide or small molecule inhibitors or agonists of the binding interaction. Also, the substrate of vertebrate *fused* can be used to isolate correlative complexing proteins. Screening assays can be designed to find lead compounds that mimic the biological activity of a native vertebrate *fused* or to find those that act as a substrate for vertebrate *fused*. Such screening assays will include assays amenable to high-throughput screening of chemical libraries, making them particularly suitable for identifying small molecule drug candidates. Such small molecule inhibitors could block the enzymatic action of fused, and thereby inhibit *hedgehog* signaling. Small molecules contemplated include synthetic organic or inorganic compounds. The assays can be performed in a variety of formats, including protein-protein binding assays, biochemical screening assays, immunoassays and cell based assays, which are well characterized in the art.

Nucleic acids which encode vertebrate *fused* or its modified forms can also be used to generate either transgenic animals or "knock out" animals which, in turn, are useful in the development and screening of therapeutically useful reagents. A transgenic animal (e.g., a mouse or rat) is an animal having cells that contain a transgene, which transgene was introduced into the animal or an ancestor of the animal at a prenatal, e.g., an embryonic stage. A transgene is a DNA that is integrated into the genome of a cell from

which a transgenic animal develops. In one embodiment. cDNA encoding vertebrate *fused* can be used to clone genomic DNA encoding vertebrate *fused* in accordance with established techniques and the genomic sequences used to generate transgenic animals that contain cells which express DNA encoding vertebrate *fused*. Methods for generating transgenic animals, particularly animals such as mice or rats, have become conventional in the art and are described, for example. in U.S. Patent Nos. 4,736,866 and 4,870,009. Typically, particular cells would be targeted for vertebrate *fused* transgene incorporation with tissue-specific enhancers. Transgenic animals that include a copy of a transgene encoding vertebrate *fused* introduced into the germ line of the animal at an embryonic stage can be used to examine the effect of increased expression of DNA encoding vertebrate *fused*. Such animals can be used as tester animals for reagents thought to confer protection from, for example, pathological conditions associated with its overexpression. For example, for basal cell carcinoma, *fused* can be overexpressed in the basal cell layer of the skin using a Keratin 5 or 14 promoter. In accordance with this facet of the invention, an animal is treated with the reagent and a reduced incidence of the pathological condition, compared to untreated animals bearing the transgene, would indicate a potential therapeutic intervention for the pathological condition.

Non-human homologues of vertebrate fused can be used to construct a vertebrate *fused* "knock out" animal which has a defective or altered gene encoding vertebrate *fused* as a result of homologous recombination between the endogenous gene encoding vertebrate *fused* and altered genomic DNA encoding vertebrate *fused* introduced into an embryonic cell of the animal. For example, cDNA encoding vertebrate *fused* can be used to clone genomic DNA encoding vertebrate *fused* in accordance with established techniques. A portion of the genomic DNA encoding vertebrate *fused* can be deleted or replaced with another gene, such as a gene encoding a selectable marker that can be used to monitor integration. Typically, several kilobases of unaltered flanking DNA (both at the 5' and 3' ends) are included in the vector [see e.g., Thomas and Capecchi, Cell, 51:503 (1987) for a description of homologous recombination vectors]. The vector is introduced into an embryonic stem cell line (e.g., by electroporation) and cells in which the introduced DNA has homologously recombined with the endogenous DNA are selected [see e.g., Li *et al.*, *Cell*, 69:915 (1992)]. The selected cells are then injected into a blastocyst of an animal (e.g., a mouse or rat) to form aggregation chimeras [see e.g., Bradley, in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E. J. Robertson, ed. (IRL, Oxford, 1987), pp. 113-152]. A chimeric embryo can then be implanted into a suitable pseudopregnant female foster animal and the embryo brought to term to create a "knock out" animal. Progeny harboring the homologously recombined DNA in their germ cells can be identified by standard techniques and used to breed animals in which all cells of the animal contain the homologously recombined DNA. Knockout animals can be characterized for instance, for their ability to defend against certain pathological conditions and for their development of pathological conditions due to absence of the vertebrate *fused* polypeptide.

As *fused* has been implicated as a universal mediator for all members of the *Hh* family (*SHh, IHh, DHh*), disease states or disorders which are associated with general *Hh* signaling, would also be treatable with *fused* and antagonists and agonists thereof. For example, *SHh* activation (e.g. *fused* agonists) has recently been promoted as a treatment for various degenerative disorders of the nervous system, *e.g.*, Parkinson's disease, memory deficits, Alzheimer's disease, Lou Gehrig's disease, Huntington's disease,

schizophrenia, stroke and drug addiction. Recent studies suggest that *Dhh* mutant males are infertile due to the failure of spermatocytes to complete their differentiation into mature sperm, Bitgood *et al.*, *Curr. Biol.* 6: 298-304 (1996); Bitgood *et al.*, *Dev. Biol.* 172: 126-138 (1995). Additionally, *fused* agonists could be used to great gut diseases, bone diseases, skin diseases, diseases of the testis, ulcers, lung diseases, diseases of the pancreas, diabetes, osteoporosis.

The presence of the protein kinase domain suggests that fused may act similarly as members of the protein kinase family in the modulation of *Hh* signaling. Protein kinases are essential elements of regulatory circuits in differentiated as well as growing cells; Preat *et al.*, *Nature* 347: 87-89 (1990). Many of these enzyme are involved in transduction of extracellular signals and operate through a cascade of phosphorylation events that amplify and disseminate the effects of a primary signal. As described earlier, *Drosophila fused* bears significant homology to other intracellular serine/threonine kinases. Many serine/threonine kinases are implicated in cell-cycle control in yeasts and in mammals, Hunter, *Cell* 50: 823-829 (1987); Dunphy & Newport, *Cell* 55: 925-928 (1988); Lee & Nurse, *Trend. Genet.* 4: 287-290 (1988).

Suppression or inhibition of *Hh* signaling is also an objective of therapeutic strategies. Since inactive *fused* has been shown to inhibit *Hh* signaling, it follows that a *fused* antagonist would also be expected to be antagonistic to *Hh* signaling. Limiting *Hh* signaling would be useful in disease states or disorders characterized by *Hh* signaling. For example, *SHh* is known to be active in Basal Cell Carcinoma; *DHh* is known to be active in spermatogenesis. Inhibitor or antagonist of *Hh* signaling would be effective therapeutics in the treatment of Basal Cell Carcinoma or male contraception, respectively.

The stimulation of *Hh* signaling is also an objective of therapeutic strategies. Activating *Hh* signaling would be useful in disease states or disorders characterized by inactive or insufficient *Hh* signaling. For example, degenerative disorders of the nervous system, *e.g.*, Parkinson's disease, memory deficits, Alzheimer's disease, Lou Gehrig's disease, Huntington's disease, schizophrenia, stroke and drug addiction. Additionally, *fused* agonists could be used to great gut diseases, bone diseases, skin diseases, diseases of the testis (including infertility), ulcers, lung diseases, diseases of the pancreas, diabetes, osteoporosis.

F. Anti-vertebrate *fused* Antibodies

The present invention further provides anti- vertebrate *fused* antibodies. Exemplary antibodies include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies.

1. Polyclonal Antibodies

The anti-vertebrate *fused* antibodies may comprise polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant. Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The immunizing agent may include the vertebrate *fused* polypeptide or a fusion protein thereof. It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins include but are not limited to keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor. Examples of adjuvants that may be employed include Freund's complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate). The immunization protocol may be

selected by one skilled in the art without undue experimentation.

2. Monoclonal Antibodies

The anti-vertebrate *fused* antibodies may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, Nature, 256:495 (1975). In a hybridoma method, a mouse, hamster, or other appropriate host animal, is typically immunized with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized *in vitro*.

The immunizing agent will typically include the vertebrate *fused* polypeptide or a fusion protein thereof. Generally, either peripheral blood lymphocytes ("PBLs") are used if cells of human origin are desired, or spleen cells or lymph node cells are used if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell [Goding, Monoclonal Antibodies: Principles and Practice, Academic Press, (1986) pp. 59-103]. Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which substances prevent the growth of HGPRT-deficient cells.

Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, California and the American Type Culture Collection, Rockville, Maryland. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies [Kozbor, J. Immunol., 133:3001 (1984); Brodeur et al., Monoclonal Antibody Production Techniques and Applications, Marcel Dekker, Inc., New York, (1987) pp. 51-63].

The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against vertebrate *fused*. Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). Such techniques and assays are known in the art. The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson and Pollard, Anal. Biochem., 107:220 (1980).

After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods [Goding, supra]. Suitable culture media for this purpose include, for example, Dulbecco's Modified Eagle's Medium and RPMI-1640 medium. Alternatively, the hybridoma cells may be grown *in vivo* as ascites in a mammal.

The monoclonal antibodies secreted by the subclones may be isolated or purified from the culture

medium or ascites fluid by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Patent No. 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences [U.S. Patent No. 4,816,567; Morrison et al., supra] or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody of the invention, or can be substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody.

The antibodies may be monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking.

In vitro methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly, Fab fragments, can be accomplished using routine techniques known in the art.

3. Humanized Antibodies

The anti-vertebrate *fused* antibodies of the invention may further comprise humanized antibodies or human antibodies. Humanized forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues that are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and

all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source that is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones et al., Nature, 321:522-525 (1986); Riechmann et al., Nature, 332:323-327 (1988); Verhoeyen et al., Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

Human antibodies can also be produced using various techniques known in the art, including phage display libraries [Hoogenboom and Winter, J. Mol. Biol., 227:381 (1991); Marks et al., J. Mol. Biol., 222:581 (1991)]. The techniques of Cole et al. and Boerner et al. are also available for the preparation of human monoclonal antibodies (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner et al., J. Immunol., 147(1):86-95 (1991)].

4. Bispecific Antibodies

Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for the vertebrate *fused*, the other one is for any other antigen, and preferably for a cell-surface protein or receptor or receptor subunit.

Methods for making bispecific antibodies are known in the art. Traditionally, the recombinant production of bispecific antibodies is based on the co-expression of two immunoglobulin heavy-chain/light-chain pairs, where the two heavy chains have different specificities [Milstein and Cuello, Nature, 305:537-539 (1983)]. Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of ten different antibody molecules, of which only one has the correct bispecific structure. The purification of the correct molecule is usually accomplished by affinity chromatography steps. Similar procedures are disclosed in WO 93/08829, published 13 May 1993, and in Traunecker et al., EMBO J., 10:3655-3659 (1991).

Antibody variable domains with the desired binding specificities (antibody-antigen combining sites) can be fused to immunoglobulin constant domain sequences. The fusion preferably is with an immunoglobulin heavy-chain constant domain, comprising at least part of the hinge, CH2, and CH3 regions. It is preferred to have the first heavy-chain constant region (CH1) containing the site necessary for light-chain binding present in at least one of the fusions. DNAs encoding the immunoglobulin heavy-chain fusions and, if desired, the immunoglobulin light chain, are inserted into separate expression vectors, and are

co-transfected into a suitable host organism. For further details of generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology, 121:210 (1986).

5. Heteroconjugate Antibodies

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells [U.S. Patent No. 4,676,980], and for treatment of HIV infection [WO 91/00360; WO 92/200373; EP 03089]. It is contemplated that the antibodies may be prepared *in vitro* using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptobutyrimidate and those disclosed, for example, in U.S. Patent No. 4,676,980.

G. Uses for anti-vertebrate *fused* Antibodies

The anti-vertebrate *fused* antibodies of the invention have various utilities. For example, anti-vertebrate *fused* antibodies may be used in diagnostic assays for vertebrate *fused*, *e.g.*, detecting its expression in specific cells, tissues, or serum. Various diagnostic assay techniques known in the art may be used, such as competitive binding assays, direct or indirect sandwich assays and immunoprecipitation assays conducted in either heterogeneous or homogeneous phases [Zola, Monoclonal Antibodies: A Manual of Techniques, CRC Press, Inc. (1987) pp. 147-158]. The antibodies used in the diagnostic assays can be labeled with a detectable moiety. The detectable moiety should be capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as ^{3}H, ^{14}C, ^{32}P, ^{35}S, or ^{125}I, a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase. Any method known in the art for conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter et al., Nature, 144:945 (1962); David *et al.*, Biochemistry, 13:1014 (1974); Pain et al., J. Immunol. Meth., 40:219 (1981); and Nygren, J. Histochem. and Cytochem., 30:407 (1982).

Anti-vertebrate *fused* antibodies also are useful for the affinity purification of vertebrate *fused* from recombinant cell culture or natural sources. In this process, the antibodies against vertebrate *fused* are immobilized on a suitable support, such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the vertebrate *fused* to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the vertebrate *fused*, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent that will release the vertebrate *fused* from the antibody.

H. *Fused* Antagonists

Several approaches may be suitably employed to create the *fused* antagonist and agonist compounds of the present invention. Any approach where the antagonist molecule can be targeted to the interior of the cell, which interferes or prevents wild type *fused* from normal operation is suitable. For example, competitive inhibitors, including mutant *fused* such as dominant negative mutant identified in the Examples,

which prevent *fused* from properly binding with other proteins necessary for *Hh* signaling. Additional properties of such antagonist or agonist molecules are readily determinable by one of ordinary skill, such as size, charge and hydrophobicity suitable for transmembrane transport.

Where mimics or other mammalian homologues of *fused* are to be identified or evaluated, the cells are exposed to the test compound and compared to positive controls which are exposed only to human *fused*, and to negative controls which were not exposed to either the compound or the natural ligand. Where antagonists or agonists of *fused* signal modulation are to be identified or evaluated, the cells are exposed to the compound of the invention in the presence of the natural ligand and compared to controls which are not exposed to the test compound.

Detection assays may by employed as a primary screen to evaluate the phosphatase inhibition/enhancing activity of the antagonist/agonist compounds of the invention. The assays may also be used to assess the relative potency of a compound by testing a range of concentrations, in a range from 100 mM to 1 pM, for example, and computing the concentration at which the amount of phosphorylation or signal transduction is reduced or increased by 50% (IC_{50}) compared to controls.

Assays can be performed to identify compounds that affect phosphorylation of *fused* substrates. Specifically, assays can be performed to identify compounds that increase the phosphorylation activity of *fused* or assays can be performed to identify compounds that decrease the phosphorylation of *fused* substrates. These assays can be performed either on whole cells themselves or on cell extracts. The assays can be performed in a variety of formats, including protein-protein binding assays, biochemical screening assays, immunoassays, cell based assays, etc. Such assay formats are well known in the art.

The screening assays of the present invention are amenable to high-throughput screening of chemical libraries, and are particularly suitable for identifying small molecule drug candidates.

(1) *Antagonist and agonist molecules*

To screen for antagonists and/or agonists of *fused* signaling, the assay mixture is incubated under conditions whereby, but for the presence of the candidate pharmacological agent, *fused* induces hedgehog signaling with a reference activity. The mixture components can be added in any order that provides for the requisite hedgehog activity. Incubation may be performed at any temperature that facilitates optimal binding, typically between about 4° and 40°C, more commonly between about 15° and 40°C. Incubation periods are likewise selected for optimal binding but also minimized to facilitate rapid, high-throughput screening, and are typically between about 0.1 and 10 hours, preferably less than 5 hours, more preferably less than 2 hours. After incubation, the effect of the candidate pharmacological agent on the *fused* signaling is determined in any convenient way. For cell-free binding-type assays, a separation step is often used to separate bound and unbound components. Separation may, for example, be effected by precipitation (e.g. TCA precipitation, immunoprecipitation, etc.), immobilization (e.g. on a solid substrate), followed by washing. The bound protein is conveniently detected by taking advantage of a detectable label attached to it, e.g. by measuring radioactive emission, optical or electron density, or by indirect detection using, *e.g.* antibody conjugates.

For example, a method of screening for suitable *fused* antagonists and/or agonists could involve the application of agents present in the *fused* activating *Gli* reporter assay described in the Examples. Such a screening assay could compare *in situ* hybridization in the presence and absence of the candidate antagonist and/or agonist in a *fused* expressing tissue as well as confirmation or absence of *fused* modulated cellular development. Typically these methods involve exposing an immobilized *fused* to a molecule suspected of binding thereto and determining binding or phosphorylation of the molecule to the immobilized *fused* and/or evaluating whether or not the molecule activates (or blocks activation of) *fused*. In order to identify such *fused* binding ligands, *fused* can be expressed on the surface of a cell and used to screen libraries of synthetic candidate compounds or naturally-occurring compounds (e.g., from endogenous sources such as serum or cells).

Suitable molecules that affect the protein-protein interaction of *fused* and its binding proteins include fragments of the latter or small molecules, *e.g.*, peptidomimetics, which will prevent interaction and proper complex formation. Such small molecules, which are usually less than 10 K molecular weight, are preferable as therapeutics since they are more likely to be permeable to cells, are less susceptible to degradation by various cellular mechanisms, and are not as apt to elicit an immune response as proteins. Small molecules include but are not limited to synthetic organic or inorganic compounds. Many pharmaceutical companies have extensive libraries of such molecules, which can be conveniently screened by using the assays of the present invention. Non-limiting examples include proteins, peptides, glycoproteins, glycopeptides, glycolipids, polysaccharides, oligosacchardies, nucleic acids, bioorganic molecules, peptidomimetics, pharmacological agents and their metabolites, transcriptional and translation control sequences, and the like.

A preferred technique for identifying molecules which bind to *fused* utilizes a chimeric substrate (*e.g.*, epitope-tagged *fused* or *fused* immunoadhesin) attached to a solid phase, such as the well of an assay plate. The binding of the candidate molecules, which are optionally labeled (e.g., radiolabeled), to the immobilized receptor can be measured. Alternatively, competition for activation of *Gli* can be measured. In screening for antagonists and/or agonists, *fused* can be exposed to a *fused* substrate followed by the putative antagonist and/or agonist, or the *fused* binding protein and antagonist and/or agonist can be added simultaneously, and the ability of the antagonist and/or agonist to block *fused* activation can be evaluated.

(2) Detection assays

The *fused* polypeptides are useful in assays for identifying lead compounds for therapeutically active agents that modulate *fused* hedgehog signaling. Specifically, lead compounds that either prevent the formation of *fused* signaling complexes or prevent or attenuate *fused* modulated hedgehog signaling (*e.g*, binding to *fused* itself or to a substrate) can be conveniently identified.

Various procedures known in the art may be used for identifying, evaluating or assaying the inhibition of activity of the *fused* proteins of the invention. As *fused* is believed to operate in a similar manner as other kinases, techniques known for use with identifying kinase/phosphatase modulators may also be employed with the present invention. In general, such assays involve exposing target cells in culture to the compounds and a) biochemically analyzing cell lysates to assess the level and/or identity of phosphorylation; or (b) scoring phenotypic or functional changes in treated cells as compared to control cells

that were not exposed to the test substance. Such screening assays are described in U.S.P. 5.602171, U.S.P. 5,710,173, WO 96/35124 and WO 96/40276.

(a) Biochemical detection techniques

Biochemical analysis techniques can be evaluated by a variety of techniques. One typical assay mixture which can be used with the present invention contains *fused* and a protein with which *fused* is normally associated (*e.g. Gli*), usually in an isolated, partially pure or pure form. One or both of these components may be *fused* to another peptide or polypeptide, which may, for example, provide or enhance protein-protein binding, improve stability under assay conditions, *etc*. In addition, one of the components usually comprises or is coupled to a detectable label. The label may provide for direct detection by measuring radioactivity, luminescence, optical or electron density, etc., or indirect detection such as an epitope tag, an enzyme, etc. The assay mixture can additionally comprise a candidate pharmacological agent, and optionally a variety of other components, such as salts, buffers, carrier proteins, e.g. albumin, detergents, protease inhibitors, nuclease inhibitors, antimicrobial agents, etc., which facilitate binding, increase stability, reduce non-specific or background interactions, or otherwise improve the efficiency or sensitivity of the assay.

The following detection methods may also be used in a cell-free system wherein cell lysate containing the signal transducing substrate molecule and *fused* is mixed with a compound of the invention. The substrate is phosphorylated by initiating the kinase reaction by the addition of adenosine triphosphate (ATP). To assess the activity of the compound, the reaction mixture may be analyzed by the SDS-PAGE technique or it may be added to substrate-specific anchoring antibody bound to a solid support, and a detection procedure as described above is performed on the separated or captured substrate to assess the presence or absence of pSer/Thr. The results are compared to those obtained with reaction mixtures to which the compound is not added. The cell-free system does not required the natural ligand or knowledge of its identity. The cell-free system does not require mixtures to which the compound is not added. The cell-free system does not require the natural ligand or knowledge of its identity. For example, Posner *et al.* (U.S.P. 5,155,031 describes the use of insulin receptor as a substrate and rat adipocytes as target cells to demonstrate the ability of pervanadate to inhibit PTP activity. Another example, Burke *et al., Biochem. Biophys. Res. Comm.* <u>204</u>: 129-134 (1994) describes the use of autophosphorylated insulin receptor and recombinant PTP1B in assessing the inhibitory activity of a phophotyrosyl mimetic.

(i) Whole cell detection

A common technique involves incubating cells with vertebrate *fused* and radiolabeled phosphate, lysing the cells, separating cellular protein components of the lysate using an SDS-polyacrylamide gel (SDS-PAGE) technique, in either one or two dimensions, and detecting the presence of phosphorylated proteins by exposing X-ray film. Detection can also be effected without using radioactive labeling. In such a technique, the protein components (e.g., separated by SDS-PAGE) are transferred to a nitrocellulose membrane where the presence of phosphorylated serine/threonines is detected using an antiphosphoserine/threonine antibody (anti-pS/T).

Alternatively, the anti-pS/T can be conjugated with an enzyme, such as horseradish peroxidase, and detected by subsequent addition of a colorimetric substrate for the enzyme. A further alternative involves

detecting the anti-PS/T by reacting with a second antibody that recognizes the anti-PS/T, this second antibody being labeled with either a radioactive moiety or an enzyme as previously described. Examples of these and similar techniques are described in Hansen *et al.*, *Electrophoresis* 14: 112-126 (1993); Campbell *et al.*, *J. Biol. Chem.* 268: 7427-7434 (1993): Donato *et al.*, *Cell Growth Diff.* 3: 258-268 (1992): Katagiri *et al.*, *J. Immunol.* 150: 585-593 (1993). Additionally, the anti-pS/T can be detected by labeling it with a radioactive substance, followed by scanning the labeled nitrocellulose to detect radioactivity or exposure of X-ray film.

(ii) Kinase assays

When the screening methods of the present invention for *fused* antagonists/agonists are carried out as an *ex vivo* assay, the target kinase (*e.g. fused*) can be a substantially purified polypeptide. The kinase substrate (*e.g.*, MBP, *Gli*) is a substantially purified substrate, which in the assay is phosphorylated in a reaction with a substantially purified phosphate source that is catalyzed by the kinase. The extent of phosphorylation is determined by measuring the amount of substrate phosphorylated in the reaction. A variety of possible substrates may be used, including the kinase itself in which instance the phosphorylation reaction measured in the assay is autophosphorylation. Exogenous substrates may also be used, including standard protein substrates such as myelin basic protein (MBP); yeast protein substrates; synthetic peptide substrates, and polymer substrates. Of these, MBP and other standard protein substrates may be regarded as preferred (see Example 10). Other substrates may be identified, however, which are superior by way of affinity for the kinase, minimal perturbation of reaction kinetics, possession of single or homogenous reaction sites, ease of handling and post-reaction recover, potential for strong signal generation, and resistance or inertness to test compounds.

Measurement of the amount of substrate phosphorylated in the *ex vivo* assay of the invention may be carried out by means of immunoassay, radioassay or other well-known methods. In an immunoassay measurement, an antibody (such as a goat or mouse anti-phosphoserine/threonine antibody) may be used which is specific for phosphorylated moieties formed during the reaction. Using well-known ELISA techniques, the phosphoserine/threonine antibody complex would itself be detected by a further antibody linked to a label capable of developing a measurable signal (as for example a fluorescent or radioactive label). Additionally, ELISA-type assays in microtitre plates may be used to test purified substrates. Peraldi *et al.*, *J. Biochem.* 285: 71-78 (1992); Schraag *et al.*, *Anal. Biochem.* 211: 233-239 (1993); Cleavland, *Anal. Biochem.* 190: 249-253 (1990); Farley, *Anal. Biochem.* 203: 151-157 (1992) and Lozaro, *Anal. Biochem.* 192: 257-261 (1991).

For example, detection schemes can measure substrate depletion during the kinase reaction. Initially, the phosphate source may be radiolabeled with an isotope such as ^{32}P or ^{33}P, and the amount of substrate phosphorylation may be measured by determining the amount of radiolabel incorporated into the substrate during the reaction. Detection may be accomplished by: (a) commercially available scintillant-containing plates and beads using a beta-counter, after adsorption to a filter or a microtitre well surface, or (b) photometric means after binding to a scintillation proximity assay bead or scintillant plate. Weemink and

Kijken. *J. Biochem. Biophs. Methods* 31: 49, 1996: Braunwalder *et al.*, *Anal. Biochem.* 234: 23 (1996); Kentrup *et al.*, *J. Biol. Chem.* 271: 3488 (1996) and Rusken *et al.*, *Meth. Enzymol.* 200: 98 (1991).

Preferably, the substrate is attached to a solid support surface by means of non-specific or, preferably, specific binding. Such attachment permits separation of the phosphorylated substrate from unincorporated, labeled phosphate source (such as adenosine triphosphate prior to signal detection. In one embodiment. the substrate may be physically immobilized prior to reaction, as through the use of Nunc™ high protein binding plate (Hanke *et al.*, *J. Biol. Chem.* 271: 695 (1996)) or Wallac ScintiStrip™ plates (Braunwalder *et al.*, *Anal. Biochem.* 234: 23 (1996). Substrate may also be immobilized after reaction by capture on, for example, P81 phophocellulose (for basic peptides), PEI/acidic molybdate resin or DEAE, or TCA precipitation onto Whatman™ 3MM paper, Tiganis *et al.*, *Arch. Biochem. Biophys.* 325: 289 (1996); Morawetz *et al.*, *Mol. Gen. Genet.* 250; 17 (1996); Budde *et al, Int J. Pharmacognosy* 33: 27 (1995) and Casnellie, *Meth. Enz.* 200: 115 (1991). Yet another possibility is the attachment of the substrate to the support surface, as by conjugation with binding partners such as glutathione and streptavidin (in the case of GST and biotin), respectively) which have been attached to the support, or via antibodies specific for the tags which are likewise attached to the support.

Further detection methods may be developed which are preferred to those described above. Especially for use in connection with high-throughput screening, it is expected that such methods would exhibit good sensitivity and specificity, extended linear range, low background signal, minimal fluctuation, compatibility with other reagents, and compatibility with automated handling systems.

The *in vivo* efficacy of the treatment of the present invention can be studied against chemically induced tumors in various rodent models. Tumor cell lines propagated in *in vitro* cell cultures can be introduced in experimental rodents, e.g. mice by injection, for example by the subcutaneous route. Techniques for chemical inducement of tumors in experimental animals are well known in the art.

(b) Biological detection techniques:

The ability of the antagonist/agonist compounds of the invention to modulate the activity *fused*, which itself modulates hedgehog signaling, may also be measured by scoring for morphological or functional changes associated with ligand binding. Any qualitative or quantitative technique known in the art may be applied for observing and measuring cellular processes which comes under the control of fused. The activity of the compounds of the invention can also be assessed in animals using experimental models of disorders caused by or related to dysfunctional *hedgehog* signaling. For example, ineffective *DHh* hedgehog signaling in mice leads to viable but sterile mice. The effects of mutant *fused* (h*fused*-DN) also affects gut development, which is regulated by *IHh* expression. Additionally, proper *SHh* signaling is critical to murine embryonic development at the notochord and floor plate, neural tube, distal limb structures, spinal column and ribs. Improper *SHh* signaling, is also correlative with cyclopia. Any of these phenotypic properties could be evaluated and quantified in a screening assay for *fused* antagonists and/or agonist. Disease states associated with overexpression of hedgehog is associated with basal cell carcinoma while inactive sonic *hedgehog* signaling leads to improper neural development.

The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosages for use in humans. The dosage of the compounds of the invention should lie within a range

of circulating concentrations with little or no toxicity. The dosage may vary within this range depending on the dosage form employed and the route of administration.

(2) *Antisense nucleotides*

Another preferred class of antagonists involves the use of gene therapy techniques, include the administration of antisense nucleotides. Applicable gene therapy techniques include single or multiple administrations of therapeutically effective DNA or mRNA. Antisense RNAs and DNAs can be used as therapeutic agents for blocking the expression of certain genes *in vivo*. Short antisense oligonucleotides can be imported into cells where they act as inhibitors, despite their low intracellular concentrations caused by restricted uptake by the cell membrane. Zamecnik *et al., Proc. Natl. Acad. Sci. USA* 83: 4143-4146 (1986). The oligonucleotides can be modified to enhance their uptake, e.g., by substituting their negatively charged phosphodiester groups by uncharged groups.

There are a variety of techniques known for introducing nucleic acids into viable cells. The techniques vary depending upon whether the nucleic acid is transferred into cultured cells *in vitro, ex vivo*, or *in vivo* in the cells of the intended host. Techniques suitable for the transfer of nucleic acid into mammalian cells in vitro include the use of liposomes, electroporation, microinjection, cell fusion, DEAE-dextran, the calcium phosphate precipitation method, etc. The currently preferred *in vivo* gene transfer techniques include transfection with viral (typically retroviral) vectors and viral coat protein-liposome mediated transfection, Dzau *et al., Trends Biotech.* 11: 205-210 (1993). In some situations it is desirable to provide the nucleic acid source with an agent that targets the target cells, such as an antibody specific for a cell surface membrane protein associated with endocytosis may be used for targeting and/or to facilitate uptake, *e.g.*, capsid proteins or fragments thereof tropic for a particular cell type, antibodies for proteins which undergo internalization in cycling, and proteins that target intracellular localization and enhance intracellular half-life. The technique of receptor-mediated endocytosis is described, for example, by Wu *et al., J. Biol. Chem.* 262: 4429-4432 (1987); Wagner *et al., Proc. Natl. Acad. Sci. USA* 87: 3410-3414 (1990). For a review of known gene marking and gene therapy protocols, see Anderson *et al., Science* 256: 808-813 (1992).

In one embodiment, *fused* antagonist and/or agonist molecules may be used to bind endogenous ligand in the cell, thereby causing the cell to be unresponsive to *fused* wild type, especially when the levels of *fused* in the cell exceed normal physiological levels. Also, it may be beneficial to bind endogenous *fused* substrates or complexing agents that are activating undesired cellular responses (such as proliferation of tumor cells).

In a further embodiment of the invention, *fused* expression may be reduced by providing *fused*-expressing cells with an amount of *fused* antisense RNA or DNA effective to reduce expression of the *fused* protein.

1. Diagnostic Uses

Another use of the compounds of the invention (*e.g.*, human and vertebrate *fused*, vertebrate *fused* variant and anti-vertebrate *fused* antibodies) described herein is to help diagnose whether a disorder is driven, to some extent, *fused* or hedgehog signaling. For example, basal cell carcinoma cells are associated with active *hedgehog* signaling.

A diagnostic assay to determine whether a particular disorder is driven by hedgehog signaling, can be carried out using the following steps: (1) culturing test cells or tissues; (2) administering a compound which can inhibit *fused* modulated *hedgehog* signaling; and (3) measuring the degree of kinase attenuation on the *fused* substrate in cell lysates or hedgehog mediated phenotypic effects in the test cells. The steps can be carried out using standard techniques in light of the present disclosure. For example, standard techniques can be used to isolate cells or tissues and culturing or *in vivo*.

Compounds of varying degree of selectivity are useful for diagnosing the role of *fused*. For example, compounds which inhibit fused in addition to another form of kinase can be used as an initial test compound to determine if one of several serine/threonine kinases drive the disorder. The selective compounds can then be used to further eliminate the possible role of the other serine/threonine kinases in driving the disorder. Test compounds should be more potent in inhibiting serine/threonine kinase activity than in exerting a cytotoxic effect (*e.g.*, an IC_{50}/LD_{50} of greater than one). The IC_{50} and LD_{50} can be measured by standard techniques, such as an MTT assay, or by measuring the amount of LDH released. The degree of IC_{50}/LD_{50} of a compound should be taken into account in evaluating the diagnostic assay. Generally, the larger the ratio the more relative the information. Appropriate controls take into account the possible cytotoxic effect of a compound of a compound, such as treating cells not associated with a cell proliferative disorder (*e.g.*, control cells) with a test compound, can also be used as part of the diagnostic assay. The diagnostic methods of the invention involve the screening for agents that modulate the effects of fused upon hedgehog signaling. Exemplary detection techniques include radioactive labeling and immunoprecipitating (U.S.P. 5,385,915).

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

EXAMPLES

Commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Rockville, Maryland.

EXAMPLE 1

Isolation of human fused cDNA clones

An expressed sequence tag (EST) DNA database (LIFESEQ™, Incyte Pharmaceuticals, Palo Alto, CA) was searched for a human homologue of the *Drosophila* segment polarity gene *fused* (SEQ ID NO 26) (Preat et al., Nature 347: 87-9 (1990)). The EST Incyte #2515662 (Fig. 2) (SEQ ID NO. 3) was identified as a potential candidate. In order to identify human cDNA libraries containing human fused clones, human cDNA libraries in pRK5 were first screened by PCR using the following primers:

h-FUSED.f (SEQ ID NO. 8) 5'-CAATACAATGGTGCTGACATCCATCAAAGGCA-3'

h-FUSED.r (SEQ ID NO. 9) 5'-'GAAGGGAGGGGTGCCTACTGCCA-3'

A fetal lung library was selected and enriched for fused cDNA clones by extension of single stranded DNA from plasmid libraries grown in dug⁻/bung⁻ host using the h-FUSED.f primer (SEQ ID NO. 8) in a reaction containing 10μl of 10x PCR Buffer (Perkin Elmer), 1μl dNTP (20 mM), 1 μl library DNA (200 ng), 0.5 ml primer, 86.5 μl H_2O and 1 μl of Amplitaq® (Perkin Elmer) added after a hot start. The reaction was denatured for 1 min. at 95°C, annealed for 1 min. at 60°C then extended for 20 min. at 72°C. DNA was extracted with phenol/$CHCl_3$, ethanol precipitated, then transformed by electroporation into DH10B host bacteria. Colonies from each transformation were plated and lifted on nylon membranes and screened with an oligo probe derived from the EST sequence of the following sequence:

h-FUSED.p (SEQ ID NO. 10) 5'-CTCCAGCTCTGGAGACATATAGAGTGGTGTGCCTTTGA-3'

The oligo probe was labeled with [γ-^{32}P]-ATP and T4 polynucleotide kinase. Filters were hybridized overnight at 42°C in 50% formamide, 5xSSC, 10xDenhardt's, 0.05M sodium phosphate (pH 6.5), 0.1% sodium pyrophosphate, 50 μg/ml of sonicated salmon sperm DNA. The filters were then rinsed in 2x SSC and washed in 0.1x SSC, 0.1% SDS then exposed to Kodak® X Ray films. Two positive clones (DNA28494 (SEQ ID NO. 6) and DNA28495 (SEQ ID NO. 4) - Figs. 4 & 5) containing an insert of approximately 5 kb were isolated and sequenced. The sequence of clone DNA28495 (SEQ ID NO. 4) contains a potential initiation methionine at position 116 followed by an open reading frame of 1944 bp (Fig. 4). However, this open reading frame (ORF) encodes a protein that is only 648 amino acids long, somewhat shorter than the 795 amino acid sequence of the Drosophila fused. Interestingly, a second open reading frame is present in the 3' region of the cDNA, from nucleotide 2295 to 4349 (Fig. 4), which suggests that the cDNA may have been improperly spliced and that an intron remains between the 2 ORFs, or correspond to an alternatively spliced variant of *fused*. The sequence of clone DNA28494 (SEQ ID NO. 6) is very similar. There is one nucleotide difference between clone DNA28495 (SEQ ID NO. 4) and clone DNA28494 (SEQ ID NO. 6) located in the first ORF at position 1863 of clone 28495 (SEQ ID NO. 4) (A vs. G) which changes the coding sequence from an Gln to a Arg at position 583. (Fig. 4). This change is likely due to an allelic variation. The first open reading frame of DNA28494 (SEQ ID NO. 6) starts at residue 115 and is followed by a 647 amino acid long open reading frame. The sequences are identical except for the one change described above at position 583 and for the last 9 residues in the first open reading frame.

EXAMPLE 2

Expression of fused clones

In order to determine the size of the protein expressed from the cDNA corresponding to DNA28495 (SEQ ID NO. 4) and DNA28494 (SEQ ID NO. 6), an HA epitope tag was inserted at the N-terminus of the protein by PCR using the following primers:

Hfus.Cla-HA.F: (SEQ ID NO. 11)

5'-CCATCGATGTACCCATACGACGTCCCAGACTACGCTGAAAAGTACCACGTGTTGGAGATG-3'

and hFus.Xba.R: (SEQ ID NO. 12)

5'-GCTCTAGACTAAGGGGCAGGTCCTGTGTTCTG-3'.

The PCR product was purified, digested with ClaI-SmaI and subcloned into the pRK5 plasmids containing

DNA28494 (SEQ ID NO. 6) and DNA28495 (SEQ ID NO. 4). DNA from each of the constructs was transfected overnight into 293 cells using the CaPO4 method (Sambrook *et al. supra*; Ausuble *et al.. supra*). After about 24 h. to 48 h. after transfection. the cells were harvested and the cell pellet was lysed in 1 ml of lysine buffer (50 mM Tris pH 8.0. 150 mM NaCl. 1 mM EDTA. 1% NP40, Aprotinin. Leupeptin. -PMSF, 1 mM NaF and 1 mM Sodium Vanadate) for 20 min at 4°C. The extract was spun for 10 min at 10K then the supernatant was transferred to a new tube and precleared with 20 µl Protein A sepharose for 1 h. The protein A sepharose was spun down and 1 µl of anti-HA antibody (5 µg. Boehringer) was added to each tube. After overnight incubation at 4°C, 30 µl of Protein G sepharose was added and the tubes incubated at 4°C for 1 hour. The protein G beads were then sun down for 1 min.. washed 3 times with lysis buffer, resuspended in 20 µl of laemli buffer in the presence of β-mercapto ethanol. Samples were denatured for 5 min. at 100°C then loaded on a 6% polyacrylamide gel. Proteins were then transferred to nitrocellulose and analyzed by Western blot using the same anti-HA antibody overnight at 1 µg/ml in blocking buffer (PBS, 0.5% Tween®, 5% non fat dry milk, 3% goat serum followed by an anti-mouse HRP. ECL was used for the detection and the membrane was exposed for 90 seconds to X-Ray films. A specific band of 150 kDa was detected in the cell pellet of cells transfected with the construct with construct corresponding to clone DNA28494 (SEQ ID NO. 6) and a specific band of approximately 100 kDa could be detected for clone DNA28495 (SEQ ID NO. 4) (Fig. 6). These bands were not present in the mock transfected control. The presence of the 150 kDa band suggests the two open reading frames of DNA28494 (SEQ ID NO. 6) can be spliced together to direct the synthesis of a large protein of 150 kDa. The absence of this band for DNA28495 (SEQ ID NO. 4) suggested that this clone apparently cannot be correctly spliced. Alternative splicing of the fused gene seems to lead to the production of several different products and may be a mechanism or regulation of *fused* activity. Specific regions at the C-terminus of the *Drosophila fused* protein is known to be required for the activity of the molecule, Therond *et al.*, *Genetics* 142: 1181-1198 (1996); Robbins *et al.. Cell* 90: 225-234 (1997). Shorter *fused* molecules truncated at the C-terminus may therefore correspond to inactive or to dominant negative forms of the molecule.

EXAMPLE 3

Northern Blots

In order to determine the best tissue source to isolate more fused cDNAs and to identify a transcript encoding a full length 150 kDa *fused* molecule, human multiple tissue northern blots I, II and fetal blot from Clontech were probed with a 1.6 kb. ClaI-AccI fragment derived from clone DNA28494 (SEQ ID NO. 6) labeled by random priming. The blots were hybridized in 50% formamide, 5x SSC. 10x Denhardt's, 0.05M Sodium phosphate (pH 6.5), 0.1% Sodium pyrophosphate. 50 mg/ml sonicated salmon sperm DNA, all in the presence of 1×10^6 cpm/ml ^{32}P-labeled probe at 42°C overnight. The blots were washed in 2x SSC at RT for 10 minutes and washed in 0.2x SSC/0.1% SDS at 42°C for 30 minutes then exposed to x-ray film overnight. Fig. 7 shows that the fused message is expressed at high levels in testis and at low levels in most other tissues. including fetal tissues. (Fig. 7).

EXAMPLE 4

PCR on different tissues to identify the correct splice form

In order to isolate a cDNA where the 2 potential ORFs were spliced together correctly, we designed the following primers flanking the potential intron and amplified various tissues including human fetal brain, brain, keratinocyte, testis, ovary, fetal liver, and lung templates.

F1 (SEQ ID NO. 13) 5'-CTGACGACACAGCAGGTTGTC-3'

R4 (SEQ ID NO. 14) 5'-CAGATGCTTCAGGATGGACAT-3'

Two microliters of each cDNA library was used as the template and PCR was done with Klentaq® polymerase. PCR was performed for 45 cycles of amplification with 94°C denaturation for 1 min., 55°C annealing for 1 min., and 68°C extensions for 2 min. One fifth of the reaction was loaded on 1% agarose gel and was Southern blotted. The blot was hybridized overnight with full-length fused probe labeled by random priming as described for the Northern blot.

A 1 kb PCR fragment was identified in fetal brain, testis and ovary. This fragment was gel-purified and subjected to direct PCR sequencing using both the F1 and R4 primers (SEQ ID NOS. 13 and 14) identified above as well a the following primers:

hf16 (SEQ ID NO. 15) 5'-AGAGTAGCAACGTCACTGC-3'

hf8 (SEQ ID NO. 16) 5'-CCTCACTGACAAGGCAGCAGG-3'

hf19 (SEQ ID NO. 17) 5'-CCCGAGGAGGCATCTGCACAG-3'

The sequence of this 1 kb fragment revealed that intron sequences were absent and that the 2 ORFs were connected together in the same reading frame. The sequence of the correctly spliced sequence is shown in Fig. 1 (SEQ ID NO. 1). The initiator ATG is present at position 161 and is followed by an ORF of 3945 nucleotides which encodes a 1315 amino acid long protein with a predicted molecular weight of 144 kDa.

The overall similarity with *Drosophila fused* (SEQ ID NO. 23) is 28% (Fig. 2). The N-terminal 263 amino acid domain of the protein containing the kinase domain is 55% homologous to the *Drosophila fused* kinase domain. The remaining 1052 amino acids portion of the protein is not appreciably homologous to other known proteins and, interestingly, is not homologous to the corresponding region in *Drosophila fused.* Interestingly, this region of non-homology includes the very C-terminus of the fly protein which appears to be required for activity, *Robbins et al., Cell* 90: 225-34 (1997); Therond *et al., Genetics* 142: 1181-98 (1996). The improperly spliced cDNAs described above may reflect alternative splicing of the *fused* gene which leads to the production of a molecule with a truncated C-terminus and may be a mechanism to regulate *fused* activity.

EXAMPLE 5

Reconstitution of the correctly spliced full length human fused

The *fused* clone DNA28495 (SEQ ID NO. 4) was subcloned from the pRK5B plasmid into pRK5.tkneo using ClaI-HindIII. PCR was performed using human testis cDNA as a template and the primers hf3 (SEQ ID NO. 18) (CAGAACTTCAGGTCCTAAAGG) and R4 (sequence see above, Example 4). PCR conditions were 45 cycles of (94°C.1 min, 46°C to 68°C temperature gradient annealing for 1 min, and 68°C. 4 min). The PCR fragment was digested with AccI and ligated in the pRK5.tkneo.fused plasmid

cut with AccI in order to replace the region containing the intron with the correct spliced form. Two subclones were sequenced between the two AccI site and had the same correct sequence.

EXAMPLE 6

In situ hybridization

E11.3 and E13.5 mouse embryos were immersion-fixed overnight at 4°C in 4% paraformaldehyde, cryoprotected overnight in 15% sucrose, embedded in O.T.C. and frozen on liquid nitrogen. Adult mouse brains were fresh frozen with powdered dry ice. P1 mouse brains, adult mouse testis and adult rat spinal cords were embedded in O.T.C. and frozen on liquid nitrogen. Sections were cut at 16 mm, and processed for *in situ* hybridization for fused by the method of Phillips *et al.*, *Science* 250: 290-294 (1990). RNA probes were labeled with ^{33}P-UTP as described by Melton *et al.*, *Nucleic Acids Res.* 12: 7035-7052 (1984). Sense and antisense probes were synthesized from a mouse fused DNA fragment using T3 and T7, respectively, corresponding to the region encoding amino acid residues 317-486 of the human sequence.

Figure 8 reveals that the mouse fused mRNA is widely distributed in *SHh* responsive tissues, including the neural tube, pre-somitic mesoderm, somites, developing limb buds and skin. Transcripts for fused were also found in the embryonic gut, testis, cartilage and muscle - Tissues that are exposed to the other members of the *Hh* protein family; Desert and Indian. In the E11-5 mouse nervous system, high levels of fused transcripts were detected throughout the forebrain, midbrain, hindbrain and spinal cord. These high levels of expression were retained in embryonic day 13.5. In both embryonic days 11.5 and 13.5, fused mRNA was detected mainly in the ventral aspect of the neural tube, in regions that are likely to be exposed to the ventral midline-derived *SHh*. By post natal day -1, widespread expression of *fused* is still maintained throughout the brain with high levels of transcripts detected in the cortex, hypocampus, ependima and choroid plexus. In the adult, low levels of *fused* expression are detected all through the brain with higher levels confined to the ependima.

The tissue distribution of *fused* and the *Hh* receptor components, *Smo* and *Ptch* show considerable overlap. All of them are initially expressed through the neural tube as well as in other *Hh* responsive tissues. However, whereas *Smo* mRNA was evenly distributed along the dorso-ventral axis, *Ptch* and fused mRNAs are found at higher levels ventrally, suggesting that they may be upregulated by *Hh*. In addition while by day E12, expression of both *Smo* and *Ptch* is found mainly in cells which are in close proximity to the ventricular zone, *fused* mRNA is still widely expressed and its levels decline only later. In the adult expression of both *Smo* and *fused* is confined to the ependima where neurogenesis continues.

Detailed analysis of fused expression in adult testis was also performed by in situ hybridization (Fig. 9). fused was found to be expressed at very high levels on stages I and II germ cells in the seminiferous tubules. Levels of fused vary in different seminiferous tubules, suggesting that its expression is regulated according to the germinal cell state of differentiation.

EXAMPLE 7

Gli Luciferase Assay

Given the low homology between d*fused* and h*fused*, it was prudent to determine whether in fact the isolated hfused is indeed a mediator of *Hh* signaling. The following assay was developed to measure the activation of the transcription factor *GLI*, the mammalian homologue of the *Drosophila cubitus interruptus*

(Ci). It has been shown that *GLI* is a transcription factor activated upon *SHh* stimulation of cells.

Nine (9) copies of a GLI binding site present in the HNF3β enhancer, (Sasaki *et al.*, *Development* 124: 1313-1322 (1997)), were introduced in front of a thymidine kinase minimal promoter driving the luciferase reporter gene in the pGL3 plasmid (Promega). The sequence of the *GLI* binding sequence was: TCGACAAGCAGGGAACACCCAAGTAGAAGCTC (p9XGliLuc) (SEQ ID NO. 19), while the negative control sequence was: TCGACAAGCAGGGAAGTGGGAAGTAGAAGCTC (p9XmGliLuc) (SEQ ID NO. 20). These constructs were cotransfected with the full length *fused* construct or with a plasmid encoding *sonic hedgehog* in C3H10T1/2 cells grown in F12, DMEM (50:50), 10% FCS heat inactivated. The day before transfection 1 x 10^5 cells per well was inoculated in 6 well plates, in 2 ml of media. The following day, 1 μg of each construct was cotransfected in duplicate with 0.025 mg ptkRenilla luciferase plasmid using lipofectamine (Gibco-BRL) in 100 μl OptiMem (with GlutaMAX) as per manufacturer's instructions for 3 hours at 37°C. Serum (20%, 1 ml) was then added to each well and the cells were incubated for 3 more hours at 37°C. Cells were then washed twice with PBS, then incubated for 48 hours at 37°C in 2 ml of media. Each well was then washed with PBS, and the cells lysed in 0.5 ml Passive Lysis Buffer (Promega) for 15 min. at room temperature on a shaker. The lysate was transferred in eppendorf tubes on ice, spun in a refrigerated centrifuge for 30 seconds and the supernatant saved on ice. For each measure, 20 μl of cell lysate was added to 100 μl of LARII (luciferase assay reagent, Promega) in a polypropylene tube and the luciferase light activity measured. The reaction was stopped by the addition of Stop and Glow buffer (Promega), mixed by pipetting up and down 3 to 5 times and *Renilla* luciferase lights activity was measured on the luminometer.

As shown in *Figure* 6, fused can induce *GLI* activity (9.5 fold) in a similar manner as *SHh* (5.5 fold) This result suggests that the *fused* gene isolated is a mediator of *SHh* signaling. An irrelevant serine-threonine kinase, *Akt*, was not active in this assay (data not shown). The fused activity is dependent on an intact kinase domain as molecules with deletion of this region (*fused* C-term) (SEQ ID NO. 27) or mutation of a conserved lysine residue at about amino acid position 33 in the ATP binding site (*fused*-DN (SEQ ID NO. 25)) were not able to activate *GLI*. Similarly, the C-terminal tail of the protein is necessary for this activity since the kinase domain alone was not active in this assay (*fused* KD) (SEQ ID NO. 24). Expression of each protein was verified by Western blot using an HA tag inserted at the N-terminus of the molecule (data not shown). These results substantiate the conclusion that the homologue of the d*fused* isolated by Applicants is indeed h*fused*. Furthermore, these results indicate that fused is capable of and sufficient for the activation of *Gli*, the major target of *SHh* signaling and is thus likely to be a direct mediator of the *SHh* signal in vertebrates.

EXAMPLE 8

Induced cyclopia in frog embryos

Introduction:

In order to demonstrate that the human fused gene is not only capable of but also required to transduce the SHh signal in vertebrates, a mutant version of fused known as fused-DN (dominant negative) having a mutation of the lysine at position 33 in the ATP binding site was created (SEQ ID NO. 25). This residue is

conserved among all kinases and is necessary for kinase activity (Hanks *et al.*, *Methods Enzymol.* 200: 38-62 (1991) and its conversion to any other residue in most cases results in the creation of dominant negative mutants.

Methods:

Plasmid Construction:

Wild type *fused* cDNA (SEQ ID NO. 1) with an HA tag inserted at the carboxy terminus was subcloned into pRK5 and a dominant negative form was generated by conversion of lysine at positive 33 to an arginine. Supercoiled plasmid DNA was prepared by Qiagen and used for injection into *Xenopus laevis* embryo.

Manipulation of Xenopus embryos:

Adult female frogs were boosted with 200 I.U. pregnant mare serum 3 days before use and with 800 I.U. of human chorionic gonadotropin the night before injection. Fresh oocytes were squeezed out from female frogs the next morning and *in vitro* fertilization of oocytes was performed by mixing oocytes with minced testis from sacrificed male frogs. Developing embryos were maintained and staged according to Nieuwkoop and Faber, Normal Table of *Xenopus laevis*, N.-H. P. Co., ed. (Amsterdam, 1967).

Fertilized eggs were dejellied with 2% cysteine (pH 7.8) for 10 minutes, washed once with distilled water and transferred to 0.1 x MBS with 5% Ficoll. Fertilized eggs were lined on injection trays in 0.1 x MBS with 5% Ficoll. Two-cell stage developing *Xenopus* embryos were injected with 200 pg of either pRK5 containing wild type *fused* (WT (SEQ ID NO. 1)) or dominant negative *fused* (DN (SEQ ID NO. 25)). Injected embryos were kept on trays for another 6 hours, after which they were transferred to 0.1 x MBS with 50 mg/ml gentamycin for 3 days until reaching Nieukwkoop stage 35 when eye development is complete.

Results:

To test whether human *fused* gene acts as a signal transducer of Hedgehog signaling, we injected wild type or dominant negative form of human *fused* in developing frog embryos. Embryos injected with 120 pg of DNA divided normally in blastula stage and gastrulate normally. While eye development was normal in wild type, *fused* (SEQ ID NO. 2) injected and mock injected embryos, about 30% (Table 1) of the embryos that were injected with *fused*-DN showed fused eye structure or two eyes connected by some pigmented retina tissue (Fig. 11A). In Table 1, 200 pg of plasmid DNA was delivered to the animal pole of 2-cell stage embryos. Each sample represents the results of at least 3 independent experiments. Embryos were scored visually for cyclopia defects.

TABLE 1

Fusion-DN Induced Cyclopia in Xenopus Embryos

Injected DNA	Normal	Cyclop	n
Hu-*fused* (SEQ ID NO. 2)	45	0	45
kinase domain (SEQ ID NO. 24)	43	0	43
C-terminus (SEQ ID NO. 27)	53	1	54
fused DN (SEQ ID NO. 25)	32	15	47
uninjected	61	0	61

The observed cyclopia phenotype is strikingly similar to the one of mouse embryos deficient in *SHh* (Chiang *et al., Nature* 383: 407-13 (1996) and of zebrafish embryos where *SHh* signaling has been blocked by overexpression of a constitutive active PKA. Hammerschmidt *et al., Genes Dev.* 10: 647-58 (1996); Ungar and Moon, *Dev. Biol.* 178: 186-91 (1996). In addition, both brain (forebrain) and gut development appeared normal at later stages of tadpole development in the *fused*-DN (SEQ ID NO. 25) injected embryos (Fig. 11B). In contrast. embryos overexpressing either wild type *fused* (SEQ ID NO. 2) or N or C-terminal terminal truncation mutants (SEQ ID NOS. 27 & 24. respectively) did not present any abnormalities.

During normal development of the Xenopus eye, the eye primordium starts as a single field expressing transcription factor Pax-6, which is a vertebrate homologue of Drosophila eyeless, Li *et al., Development,* 124: 603-15 (1997). At the neurula stage, this eye field is separated into two eye primordia due to an inhibiting signal from prechordal mesoderm. It has been further demonstrated that *SHh* is the prechordal mesoderm derived signal that is responsible for the inhibition of Pax-6 expression in the midline of the eyefield.

To further understand how overexpression of *fused*-DN (SEQ ID NO: 25) induced a fused eye in Xenopus embryos, whole mount in situ hybridization was performed in order to determine the expression pattern of Pax-6 in injected embryos. As shown in Figure 11C, Pax-6 expression in embryos injected with *fused*-DN (SEQ ID NO: 25) remains as a single field (Fig. 11D). Thus *fused*-DN (SEQ ID NO: 25) induces a cyclopia phenotype by most likely preventing *SHh* from inhibiting Pax-6 expression in the midline of the eyefield.

EXAMPLE 9

Rescue of fused-DN (SEQ ID NO. 25) Injected *Xenopus* Embryos by Gli

SHh expression in early floor plate cells is induced by *SHh* produced by the notochord. To test whether *SHh* expression in the floor plate will also be inhibited when *SHh* signaling is blocked, early neurula stage embryos injected with fused-DN or wild-type constructs were stained for *SHh* expression (See Example 8 for procedure). *SHh* expression in floor plate cells or early neurula stage embryos was completely suppressed in 26 out of 28 embryos injected when the mutated fused is overexpressed (Table 2, Figure 11C, left embryo), while the expression of *SHh* was unaffected in control embryos (Fig. 6E, right embryo). Table 2 represents scored data from three independent experiments. 100 pg of fused-DN, 100 pg of fused-wt or 50 pg of Gli-1 plasmid were injected in 2-cell stage embryos. Embryos were harvested at early neurula stage for *SHh* staining.

TABLE 2

Wild type *fused* and Gli rescue SHh expression in floor plate when coexpressed with fused-DN

	SHh staining	percentage
fused-DN	2/28	7%
fused-DN + *fused* WT	20/24	83%
fused-DN + Gli	36/36	100

To confirm that this phenotype was due to specific inhibition of the *SHh* signaling pathway in the floor plate, we attempted to rescue the phenotype by coinjection of wt *fused* RNA with *fused*-DN RNA in a 1:1 ratio. Table 2 shows that more than 80% of the embryos coinjected with wt *fused* and *fused*-DN RNAs show normal *SHh* staining in the floor plate. This demonstrates that *SHh* expression in *fused*-DN injected embryos is specifically blocked by inhibition of endogenous fused activity.

To further demonstrate that the observed phenotype of *fused*-DN are due to disruption of the *SHh* signal cascade and to confirm that h*fused* works upstream of *Gli* in this pathway, we asked whether the overexpression of *Gli* can also rescue the phenotype of *Xenopus* embryos injected with *fused*-DN. As shown in Table 2. the rescue of SHh expression in the floor plate of fused-DN injected embryos is complete when *Gli* is overexpressed. Taken together. these findings are consistent with Applicants hypothesis that vertebrate fused functions in the *SHh* pathway and that is a necessary mediator in the *SHh* signal transduction pathway, which acts upstream of *Gli*.

EXAMPLE 10

Immunoprecipitations and *In Vitro* Kinase Assay

To directly determine whether h*fused* has kinase activity, *fused* (SEQ ID NO. 2), *fused*-DN (SEQ ID NO. 25) and *fused*-kd (SEQ ID NO. 24) cDNAs were tagged with the influenza HA epitope tag and transiently transfected into 293 cells. Immunoprecipitates were tested for kinase activity in the presence of myelin basic protein (MBP) and [γ-^{32}P]-ATP. The amount of 32P incorporated into MBP was determined after SDS-PAGE and found to be was about 3 times higher than in *fused*-KD (SEQ ID NO. 24) and 2 times higher in wt *fused* (SEQ ID NO. 2) containing extracts compared to controls. while mutation of Lys33 to Arg (*fused*-DN (SEQ ID NO. 25)) neutralizes the activity (Fig. 12).

For immunoprecipitation experiments human embryonic kidney 293 cells were transiently transfected with the various expression plasmids. After 24 hours. the transfected cells were collected and lysed for 20 min. at 4°C in 1 ml of lysis buffer (50 mM Tris. pH 8.0), 150 mM NaCl. 1 mM EDTA, 1 mM sodium fluoride. 1 mM sodium orthovanadate, 1 mM PMSF and protease inhibitors (Complete, Boehringer Mannheim) containing 1% NP-40, 0.5% deoxycholic acid. Cell debris was removed by centrifugation for 10 min. at 10,000 rpm and the sodium chloride concentration of the cell lysates was increased to 250 mM. The supernatant was precleared for 1 hour with 20 µl Protein A Sepharose (Pharmacia). Lysates were immunoprecipitated using anti-HA antibodies followed by Protein A Sepharose. The beads were washed twice with lysis buffer containing 250 mM sodium chloride. twice with lysis buffer containing 1 M sodium chloride, and then twice with kinase assay buffer (20 mM HEPES, pH 7.6), 1 mM DTT. 1 mM NaF and 1 mM sodium orthovanadate). After the last wash, the beads were resuspended in 20 µl kinase assay buffer supplemented with 10 mCi [γ-^{32}P]-ATP. 20 mM β-glycerophosphate. 20 mM PNPP, 20 mM $MgCl_2$. 1 mM EGTA, 100 µM cold ATP and 0.5 mg/ml Myelin Basic Protein (Sigma). and incubated for 20 min. at 37°C. Reactions were stopped with 20 µl SDS-sample buffer. run on a denaturing 4-20% SDS polyacrylamide gel, and analyzed by phosphoimager.

EXAMPLE 11

Expression of *fused* in *E. coli*

The DNA sequence encoding human *fused* is initially amplified using selected PCR primers. The primers should contain restriction enzyme sites that correspond to the restriction enzyme sites on the selected expression vector. A variety of expression vectors may be employed. An example of a suitable vector is pBR322 (derived from *E. coli*; see Bolivar *et al.*, *Gene*, 2:95 (1977)) which contains genes for ampicillin and tetracycline resistance. The vector is digested with restriction enzyme and dephosphorylated. The PCR amplified sequences are then ligated into the vector. The vector will preferably include sequences that encode for an antibiotic resistance gene, a trp promoter, a polyhis leader (including the first six STII codons, polyhis sequence, and enterokinase cleavage site), the vertebrate fused coding region, lambda transcriptional terminator, and an argU gene.

The ligation mixture is then used to transform a selected *E. coli* strain using the methods described in Sambrook et al., supra. Transformants are identified by their ability to grow on LB plates and antibiotic resistant colonies are then selected. Plasmid DNA can be isolated and confirmed by restriction analysis and DNA sequencing.

Selected clones can be grown overnight in liquid culture medium such as LB broth supplemented with antibiotics. The overnight culture may subsequently be used to inoculate a larger scale culture. The cells are then grown to a desired optical density, during which the expression promoter is turned on.

After culturing the cells for several more hours, the cells can be harvested by centrifugation. The cell pellet obtained by the centrifugation can be solubilized using various agents known in the art, and the solubilized vertebrate *fused* protein can then be purified using a metal chelating column under conditions that allow tight binding of the protein.

EXAMPLE 12

Expression of *fused* in mammalian cells

The vector, pRK5 (see EP 307,247, published March 15, 1989), is employed as the expression vector. Optionally, the vertebrate fused DNA is ligated into pKK5 with selected restriction enzymes to allow insertion of the vertebrate fused DNA using ligation methods such as described in Sambrook et al., supra. The resulting vector is called pRK5-fused.

In one embodiment, the selected host cells may be 293 cells. Human 293 cells (ATCC CCL 1573) are grown to confluence in tissue culture plates in medium such as DMEM supplemented with fetal calf serum and optionally, nutrient components and/or antibiotics. About 10 μg pRK5-*fused* DNA is mixed with about 1 μg DNA encoding the VA RNA gene [Thimmappaya *et al.*, *Cell*, 31:543 (1982)] and dissolved in 500 μl of 1 mM Tris-HCl, 0.1 mM EDTA, 0.227 M $CaCl_2$. To this mixture is added, dropwise, 500 μl of 50 mM HEPES (pH 7.35), 280 mM NaCl, 1.5 mM $NaPO_4$, and a precipitate is allowed to form for 10 minutes at 25°C. The precipitate is suspended and added to the 293 cells and allowed to settle for about four hours at 37°C. The culture medium is aspirated off and 2 ml of 20% glycerol in PBS is added for 30 seconds. The 293 cells are then washed with serum free medium, fresh medium is added and the cells are incubated for about 5 days.

```
ttt acc cat tcg cat gtc gtc tct ctt gtg agt gca gca 2569
Phe Thr His Ser His Val Val Ser Leu Val Ser Ala Ala
                795                 800

gcc tgt cta ttg gga cag ctt ggt cag caa ggg gtg acc 2608
Ala Cys Leu Leu Gly Gln Leu Gly Gln Gln Gly Val Thr
    805                 810                 815

ttt gac ctc cag ccc atg gaa tgg atg gct gca gcc aca 2647
Phe Asp Leu Gln Pro Met Glu Trp Met Ala Ala Ala Thr
            820                 825

cat gcc ttg tct gcc cct gca gag gtt cgg ttg act cca 2686
His Ala Leu Ser Ala Pro Ala Glu Val Arg Leu Thr Pro
830                 835                 840

cca ggt agt tgt gga ttc tat gat ggc ctc ctt atc ctt 2725
Pro Gly Ser Cys Gly Phe Tyr Asp Gly Leu Leu Ile Leu
        845                 850                 855

ctg ttg cag ctc ctc act gag cag ggg aag gct agc cta 2764
Leu Leu Gln Leu Leu Thr Glu Gln Gly Lys Ala Ser Leu
                860                 865

atc agg gat atg tcc agt tca gaa atg tgg acc gtt ttg 2803
Ile Arg Asp Met Ser Ser Ser Glu Met Trp Thr Val Leu
    870                 875                 880

tgg cac cgc ttc tcc atg gtc ctg agg ctc ccc gag gag 2842
Trp His Arg Phe Ser Met Val Leu Arg Leu Pro Glu Glu
            885                 890

gca tct gca cag gaa ggg gag ctt tcg cta tcc agt cca 2881
Ala Ser Ala Gln Glu Gly Glu Leu Ser Leu Ser Ser Pro
895                 900                 905

cca agc cct gag cca gac tgg aca ctg att tct ccc cag 2920
Pro Ser Pro Glu Pro Asp Trp Thr Leu Ile Ser Pro Gln
        910                 915                 920

ggc atg gca gcc ctg ctg agc ctg gcc atg gcc acc ttt 2959
Gly Met Ala Ala Leu Leu Ser Leu Ala Met Ala Thr Phe
                925                 930

acc cag gag ccc cag tta tgc ctg agc tgc ctg tcc cag 2998
Thr Gln Glu Pro Gln Leu Cys Leu Ser Cys Leu Ser Gln
    935                 940                 945

cat gga agt atc ctc atg tcc atc ctg aag cat ctg ctt 3037
His Gly Ser Ile Leu Met Ser Ile Leu Lys His Leu Leu
            950                 955

tgc ccc agc ttc ctg aat caa ctg cgc cag gcg cct cat 3076
Cys Pro Ser Phe Leu Asn Gln Leu Arg Gln Ala Pro His
960                 965                 970

ggg tct gag ttt ctc cct gtc gtg gtg ctc tct gtc tgc 3115
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                600                     605

tac tcc agc ttg ctg acg aca cag cag gtt gtc ttg gat 2023
Tyr Ser Ser Leu Leu Thr Thr Gln Gln Val Val Leu Asp
    610                 615                 620

ggg ctc ctt cat ggc ttg aca gtt cca cag ctc cct gtc 2062
Gly Leu Leu His Gly Leu Thr Val Pro Gln Leu Pro Val
            625                 630

cac act ccc caa gga gcc ccg caa gtg agc cag cca ctg 2101
His Thr Pro Gln Gly Ala Pro Gln Val Ser Gln Pro Leu
635                 640                 645

cga gag cag agt gag gat ata cct gga gcc att tcc tct 2140
Arg Glu Gln Ser Glu Asp Ile Pro Gly Ala Ile Ser Ser
        650                 655                 660

gcc ctg gca gcc ata tgc act gct cct gtg gga ctg ccc 2179
Ala Leu Ala Ala Ile Cys Thr Ala Pro Val Gly Leu Pro
                665                 670

gac tgc tgg gat gcc aag gag cag gtc tgt tgg cat ttg 2218
Asp Cys Trp Asp Ala Lys Glu Gln Val Cys Trp His Leu
    675                 680                 685

gca aat cag cta act gaa gac agc agc cag ctc agg cca 2257
Ala Asn Gln Leu Thr Glu Asp Ser Ser Gln Leu Arg Pro
            690                 695

tcc ctc atc tct ggc ctg cag cat ccc atc ctg tgc ctg 2296
Ser Leu Ile Ser Gly Leu Gln His Pro Ile Leu Cys Leu
700                 705                 710

cac ctt ctc aag gtt cta tac tcc tgc tgc ctt gtc agt 2335
His Leu Leu Lys Val Leu Tyr Ser Cys Cys Leu Val Ser
        715                 720                 725

gag ggc ctg tgc cgt ctt ctg ggg cag gag ccc ctg gcc 2374
Glu Gly Leu Cys Arg Leu Leu Gly Gln Glu Pro Leu Ala
                730                 735

ttg gaa tcc ctg ttt atg ttg att cag ggc aag gta aaa 2413
Leu Glu Ser Leu Phe Met Leu Ile Gln Gly Lys Val Lys
    740                 745                 750

gta gta gat tgg gaa gag tct act gaa gtg aca ctc tac 2452
Val Val Asp Trp Glu Glu Ser Thr Glu Val Thr Leu Tyr
            755                 760

ttc ctc tcc ctt ctt gtc ttt cgg ctc caa aac ctg cct 2491
Phe Leu Ser Leu Leu Val Phe Arg Leu Gln Asn Leu Pro
765                 770                 775

tgt gga atg gag aag cta ggc agt gac gtt gct act ctc 2530
Cys Gly Met Glu Lys Leu Gly Ser Asp Val Ala Thr Leu
        780                 785                 790
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Pro Asp Ser Asp Asn Glu Trp Gln His Leu Leu Glu Thr
    415                 420                 425

act gag cct gtg cct att caa ctg aag gct cct ctc acc 1477
Thr Glu Pro Val Pro Ile Gln Leu Lys Ala Pro Leu Thr
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Leu Leu Cys Asn Pro Asp Phe Cys Gln Arg Ile Gln Ser
440                 445                 450

cag ctg cat gaa gct gga ggg cag atc ctg aaa ggc atc 1555
Gln Leu His Glu Ala Gly Gly Gln Ile Leu Lys Gly Ile
        455                 460                     465

ttg gag ggt gct tcc cac atc ctg cct gca ttc cgg gtc 1594
Leu Glu Gly Ala Ser His Ile Leu Pro Ala Phe Arg Val
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ctg agc agt ctt ctc tcc agc tgc agt gat tct gtt gcc 1633
Leu Ser Ser Leu Leu Ser Ser Cys Ser Asp Ser Val Ala
    480                 485                 490

ttg tat tcc ttc tgc cgg gag gca ggg ctt cct ggg ctg 1672
Leu Tyr Ser Phe Cys Arg Glu Ala Gly Leu Pro Gly Leu
            495                 500

ctg ctg agt cta ctc agg cac agt cag gag agc aac agc 1711
Leu Leu Ser Leu Leu Arg His Ser Gln Glu Ser Asn Ser
505                 510                 515

ctc cag cag caa tct tgg tat ggg acc ttc tta cag gac 1750
Leu Gln Gln Gln Ser Trp Tyr Gly Thr Phe Leu Gln Asp
        520                 525                 530

ctg atg gct gtg att cag gcc tac ttt gcc tgt acc ttc 1789
Leu Met Ala Val Ile Gln Ala Tyr Phe Ala Cys Thr Phe
                535                 540

aat ctg gag agg agc cag aca agt gac agc ctg cag gtg 1828
Asn Leu Glu Arg Ser Gln Thr Ser Asp Ser Leu Gln Val
    545                 550                 555

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Phe Gln Glu Ala Ala Asn Leu Phe Leu Asp Leu Leu Gly
            560                 565

aaa ctg ctg gcc caa cca gat gac tct gag cag act ttg 1906
Lys Leu Leu Ala Gln Pro Asp Asp Ser Glu Gln Thr Leu
570                 575                 580

cgg agg gac agc ctt atg tgc ttt act gtc ctg tgc gaa 1945
Arg Arg Asp Ser Leu Met Cys Phe Thr Val Leu Cys Glu
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            235                 240

tgg cca gac ctc tta tat cac ccc ttt att gct ggt cat 931
Trp Pro Asp Leu Leu Tyr His Pro Phe Ile Ala Gly His
245                 250                 255

gtc acc ata ata act gag cca gca ggc cca gat ttg ggg 970
Val Thr Ile Ile Thr Glu Pro Ala Gly Pro Asp Leu Gly
        260                 265                 270

acc cca ttc acc agc cgc cta ccc cca gaa ctt cag gtc 1009
Thr Pro Phe Thr Ser Arg Leu Pro Pro Glu Leu Gln Val
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cta aag gac gaa cag gcc cat cgg ttg gcc ccc aag ggt 1048
Leu Lys Asp Glu Gln Ala His Arg Leu Ala Pro Lys Gly
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aat cag tct cgc atc ttg act cag gcc tat aaa cgc atg 1087
Asn Gln Ser Arg Ile Leu Thr Gln Ala Tyr Lys Arg Met
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gct gag gag gcc atg cag aag aaa cat cag aac aca gga 1126
Ala Glu Glu Ala Met Gln Lys Lys His Gln Asn Thr Gly
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cct gcc ctt gag caa gag gac aag acc agc aag gtg gct 1165
Pro Ala Leu Glu Gln Glu Asp Lys Thr Ser Lys Val Ala
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cct ggc aca gcc cct ctg ccc aga ctc ggg gcc act cct 1204
Pro Gly Thr Ala Pro Leu Pro Arg Leu Gly Ala Thr Pro
                340                 345

cag gaa tca agc ctc ctg gcc ggg atc tta gcc tca gaa 1243
Gln Glu Ser Ser Leu Leu Ala Gly Ile Leu Ala Ser Glu
    350                 355                 360

ttg aag agc agc tgg gct aaa tca ggg act gga gag gtg 1282
Leu Lys Ser Ser Trp Ala Lys Ser Gly Thr Gly Glu Val
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ccc tct gca cct cgg gaa aac cgg acc acc cca gat tgt 1321
Pro Ser Ala Pro Arg Glu Asn Arg Thr Thr Pro Asp Cys
375                 380                 385

gaa cga gca ttc cca gag gag agg cca gag gtg ctg ggc 1360
Glu Arg Ala Phe Pro Glu Glu Arg Pro Glu Val Leu Gly
        390                 395                 400

cag cgg agc act gat gta gtg gac ctg gaa aat gag gag 1399
Gln Arg Ser Thr Asp Val Val Asp Leu Glu Asn Glu Glu
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    Arg Glu Ile Glu Ile Met Arg Gly Leu Arg His Pro Asn
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    Ile Val His Met Leu Asp Ser Phe Glu Thr Asp Lys Glu
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    gtg gtg gtg gtg aca gac tat gct gag gga gag ctc ttt 424
    Val Val Val Val Thr Asp Tyr Ala Glu Gly Glu Leu Phe
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    Gln Ile Leu Glu Asp Asp Gly Lys Leu Pro Glu Asp Gln
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    Val Gln Ala Ile Ala Ala Gln Leu Val Ser Ala Leu Tyr
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    Tyr Leu His Ser His Arg Ile Leu His Arg Asp Met Lys
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    cct cag aac atc ctc ctc gcc aag ggt ggt ggc atc aag 580
    Pro Gln Asn Ile Leu Leu Ala Lys Gly Gly Gly Ile Lys
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    Leu Cys Asp Phe Gly Phe Ala Arg Ala Met Ser Thr Asn
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    aca atg gtg ctg aca tcc atc aaa ggc aca cca ctc tat 658
    Thr Met Val Leu Thr Ser Ile Lys Gly Thr Pro Leu Tyr
        155                 160                 165

    atg tct cca gag ctg gtg gag gag cga cca tac gac cac 697
    Met Ser Pro Glu Leu Val Glu Glu Arg Pro Tyr Asp His
                170                 175

    aca gcg gac ctc tgg tct gtt ggc tgc ata cta tat gaa 736
    Thr Ala Asp Leu Trp Ser Val Gly Cys Ile Leu Tyr Glu
    180                 185                 190

    ctg gca gta ggc acc cct ccc ttc tat gct aca agc atc 775
    Leu Ala Val Gly Thr Pro Pro Phe Tyr Ala Thr Ser Ile
            195                 200                 205

    ttt cag ctg gtc agc ctc att ctc aag gac cct gtg cgc 814
    Phe Gln Leu Val Ser Leu Ile Leu Lys Asp Pro Val Arg
                    210                 215

    tgg ccc tca acc atc agt ccc tgc ttt aag aac ttc ctg 853
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gcgtccgccc acgcgtccgg ggcgtcccag atgttgtgga actgtccctg 100

gatctatagc tcttcaccgt ctctactttc ttccttctaa gagatcctga 150

aacctctgtc atg gaa aag tac cac gtg ttg gag atg att 190
Met Glu Lys Tyr His Val Leu Glu Met Ile
1 5 10

gga gaa ggc tct ttt ggg agg gtg tac aag ggt cga aga 229
Gly Glu Gly Ser Phe Gly Arg Val Tyr Lys Gly Arg Arg
15 20

aaa tac agt gct cag gtc gtg gcc ctg aag ttc atc cca 268
Lys Tyr Ser Ala Gln Val Val Ala Leu Lys Phe Ile Pro
25 30 35

aaa ttg ggg cgc tca gag aag gag ctg agg aat ttg caa 307
Lys Leu Gly Arg Ser Glu Lys Glu Leu Arg Asn Leu Gln

FIG. 12

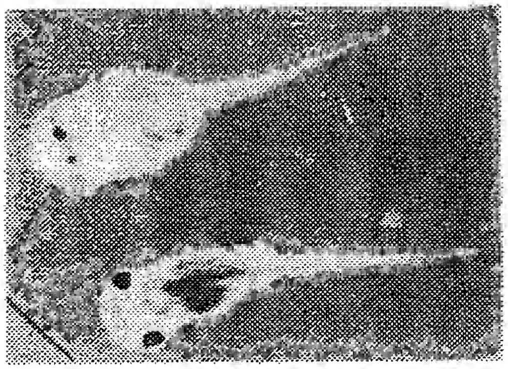


FIG. 11A

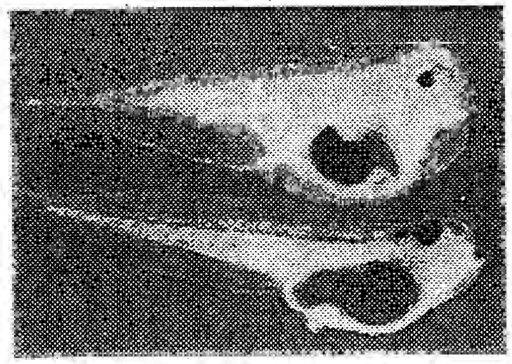


FIG. 11B

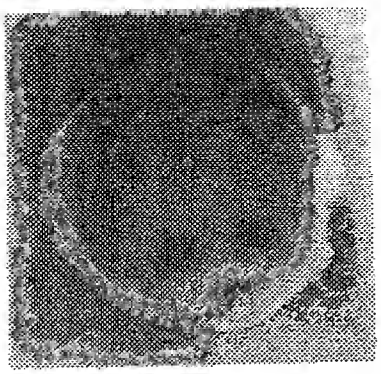


FIG. 11C

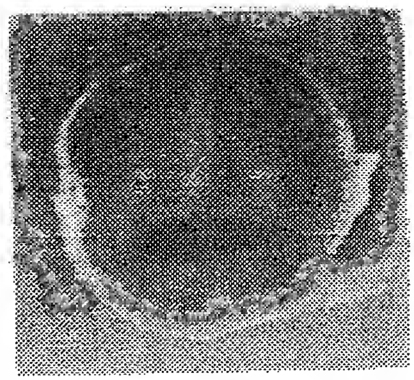


FIG. 11D

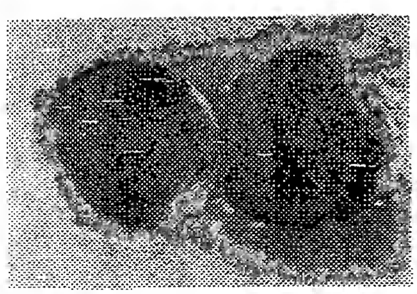
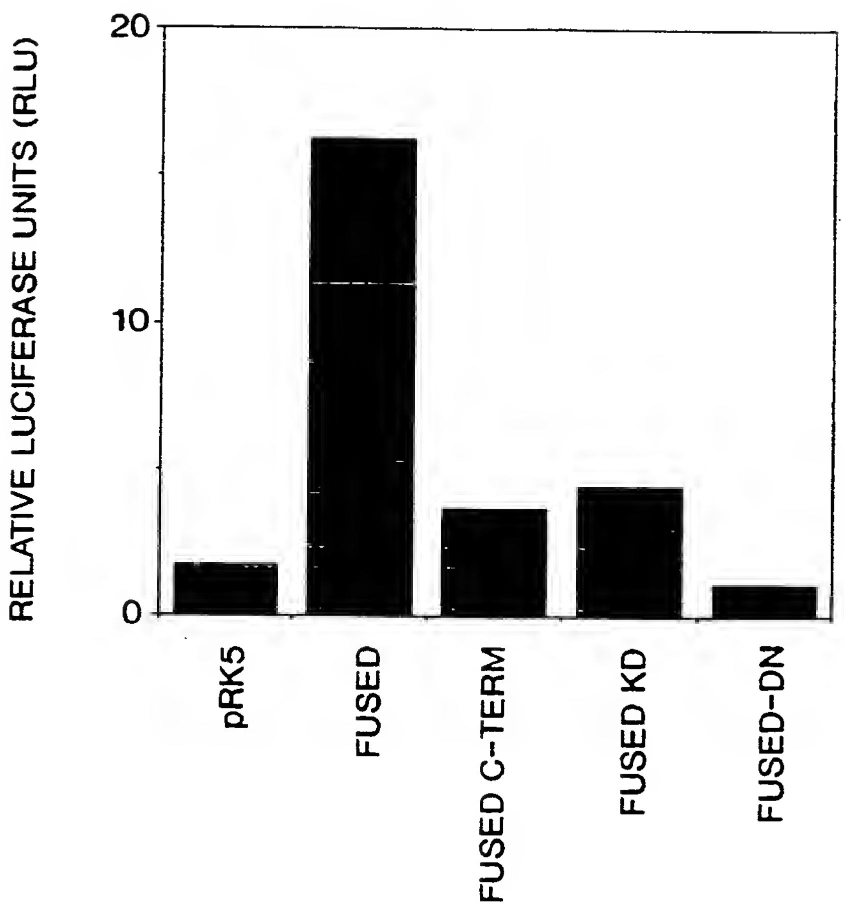


FIG. 11E

FIG. 10B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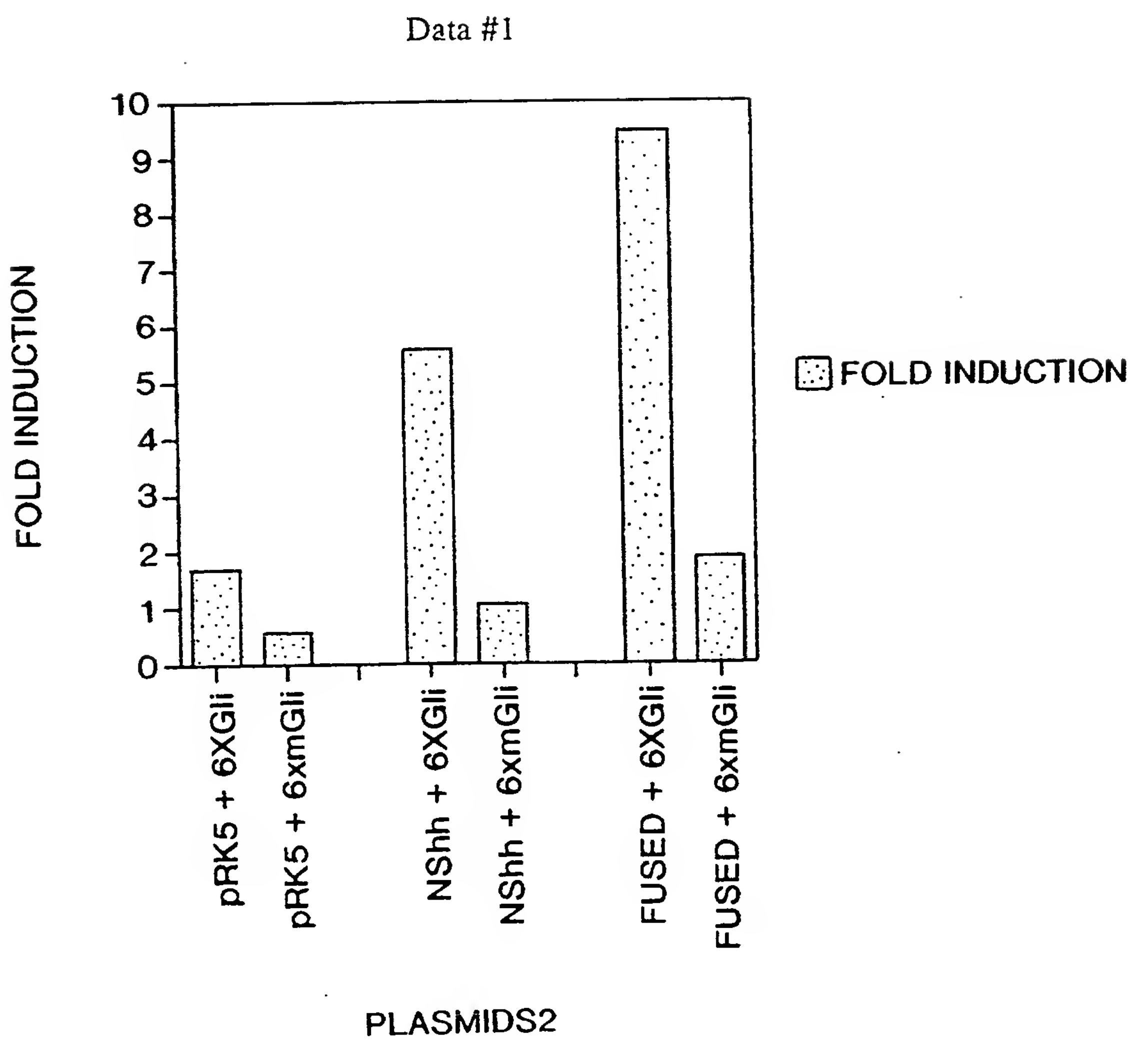
pRK5
FUSED
FUSED C-TERM
FUSED KD
FUSED-DN

FIG. 10A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8
7
6
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4
3
2
1
0
FOLD INDUCTION
FOLD INDUCTION
pRK5 + 6XGli
pRK5 + 6xmGli
NShh + 6XGli
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FUSED + 6XGli
FUSED + 6xmGli
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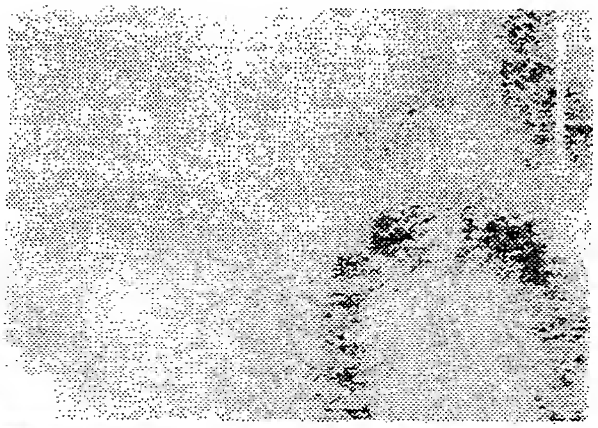

FIG. 9C

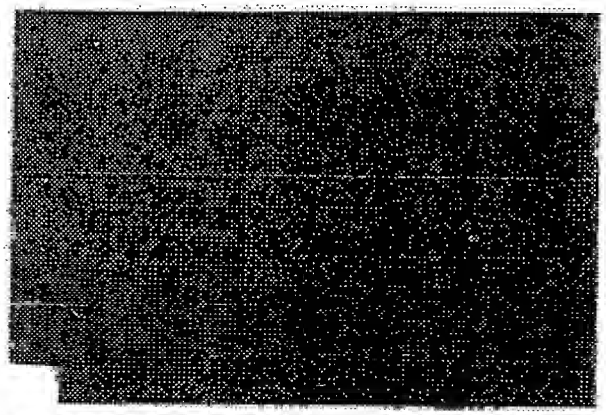

FIG. 9B

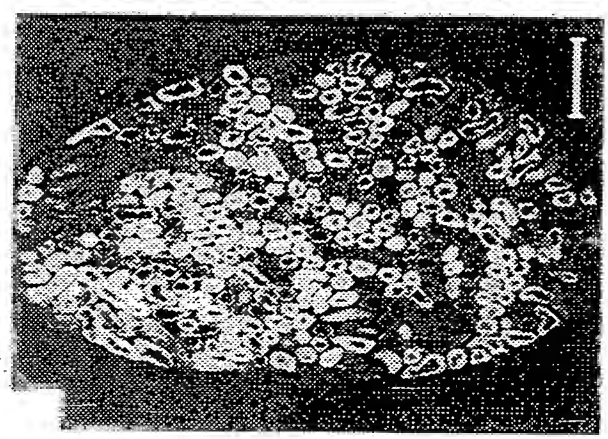

FIG. 9A

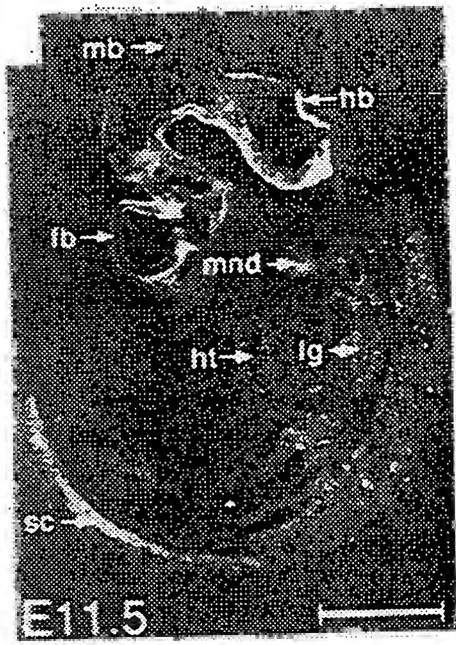

FIG. 8A

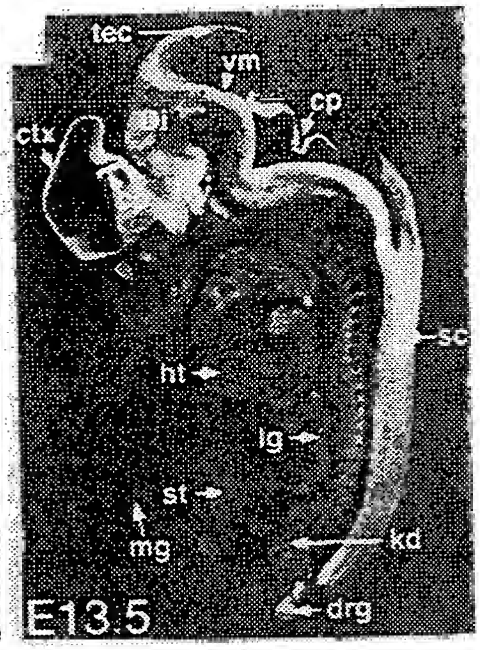

FIG. 8B

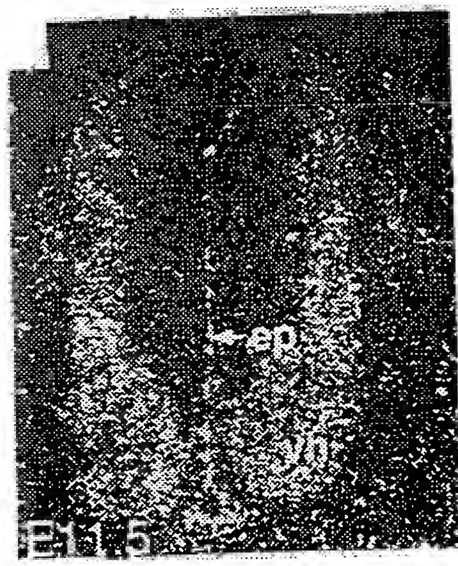

FIG. 8C

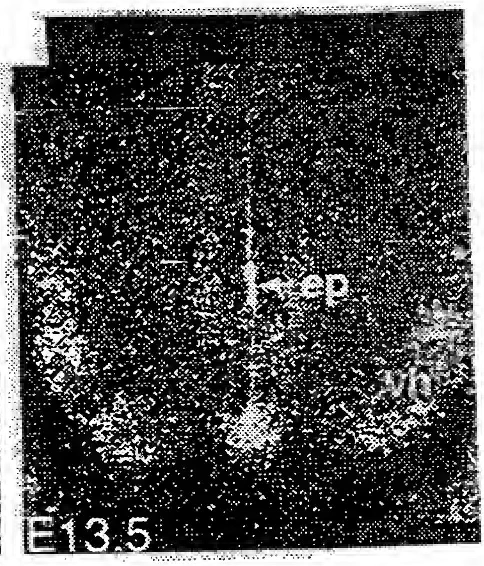

FIG. 8D

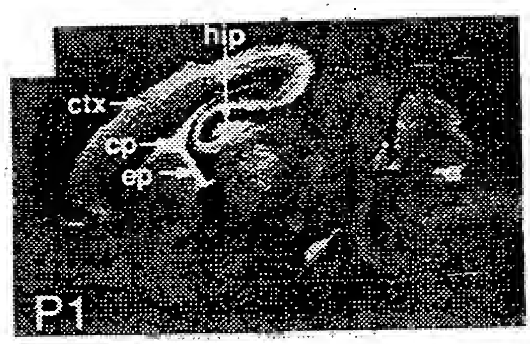

FIG. 8E

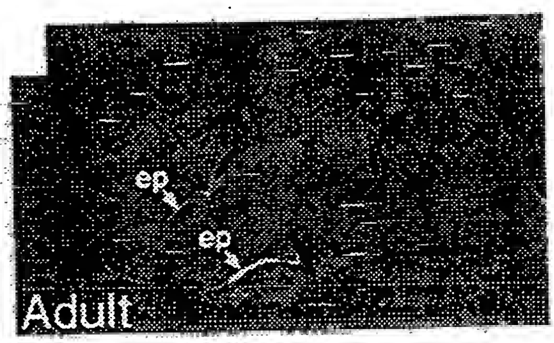

FIG. 8F

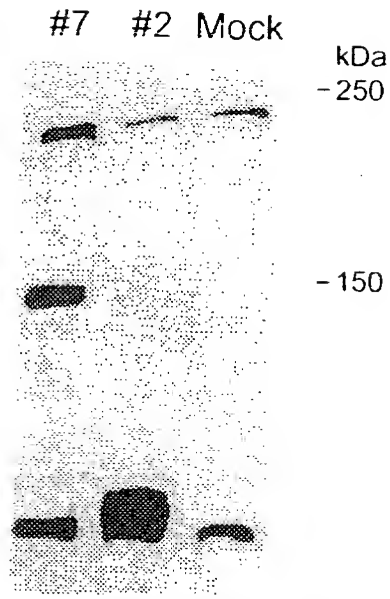
#7
#2
Mock
kDa
250
150

FIG. 6

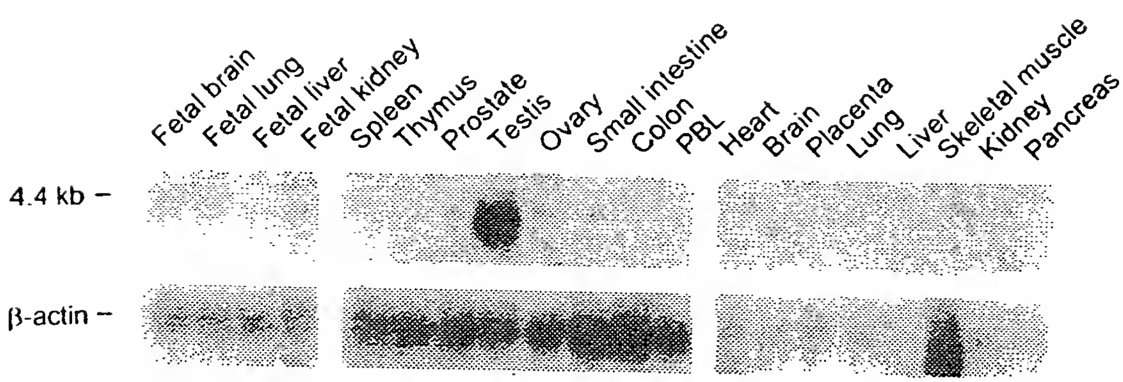
Fetal brain
Fetal lung
Fetal liver
Fetal kidney
Spleen
Thymus
Prostate
Testis
Ovary
Small intestine
Colon
PBL
Heart
Brain
Placenta
Lung
Liver
Skeletal muscle
Kidney
Pancreas
4.4 kb
β-actin

FIG. 7

```
4701 CTCCCAGATG CAGGATGTTT TCAACCAGTA AATTTTATTG CTGTTGGTGC CAGAGAAGAG TCCTTTCTTC TCTACATCCA GGGGCCTTTT CTCCAATAAT
     GAGGGTCTAC GTCCTACAAA AGTTGGTCAT TTAAAATAAC GACAACCACG GTCTCTTCTC AGGAAAGAAG AGATGTAGGT CCCCGGAAAA GAGGTTATTA

4801 GTGCCTTTAA CTCTAGGGAC CTGCCTCACG GACCTTAGGG AAAAACCTCA ACCTGAAAGA TCTCTTCCTT TCTGGAGCTC CTTTAATCTT CCCAGCAGGT
     CACGGAAATT GAGATCCCTG GACGGAGTGC CTGGAATCCC TTTTTGGAGT TGGACTTTCT AGAGAAGGAA AGACCTCGAG GAAATTAGAA GGGTCGTCCA

4901 TTTTGCCTTA GACGTGCTGG CCCCAGGACA GTGATGAAGA CAGAGCCTGT CTCAGCTCTA GGCTGTGGGG ATCAATGCCA TCAGTCCCTG TTATTGAGGG
     AAAACGGAAT CTGCACGACC GGGGTCCTGT CACTACTTCT GTCTCGGACA GAGTCGAGAT CCGACACCCC TAGTTACGGT AGTCAGGGAC AATAACTCCC

5001 ATTATCCCTT AGCCAACATT CCTATCTGTG GGTGGGCGTG GAGAGTGTAT CTTTTTTTGG GGTGTGTGTG TATATGTGTG TGTGTATGTG TGTGTGTGTT
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     ATTATCAAGA CAAACATTTG AGAAAATTAT TTTCAACACG GAGTGGTATG AACTTCGAGG GTCCTGTTCC CAACTCTCCG AGTTGGGGAG AAAGTCGAAG

5201 TATGTGGTGT TGGAGGTGCT GGTATCGTGT TCACACAAAA AAAAAAAAAA AA
     ATACACCACA ACCTCCACGA CCATAGCACA AGTGTGTTTT TTTTTTTTTT TT
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FIG. 5F

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     GGCTGGAGTC CCTGAGTCTT CAACGTCGGG TAGACGACGT CCAGACGACG ATGGTAGAAG GCAACTACGT TCACCTCGAC GGGTAGTCGG AAGAGTGTGC

3701 CCTGGCCCTC ATGGATCCCA CCTCTCTCAA CCAGTTTGTG AACACAGTGT CTGCCTCCCC TAGAACCATC GTCTCGTTTC TCTCAGTTGC CCTCCTGAGT
     GGACCGGGAG TACCTAGGGT GGAGAGAGTT GGTCAAACAC TTGTGTCACA GACGGAGGGG ATCTTGGTAG CAGAGCAAAG AGAGTCAACG GGAGGACTCA

3801 GACCAGCCAC TGTTGACCTC CGACCTTCTC TCTCTGCTGG CCCATACTGC CAGGGTCCTG TCTCCCAGCC ACTTGTCCTT TATCCAAGAG CTTCTGGCTG
     CTGGTCGGTG ACAACTGGAG GCTGGAAGAG AGAGACGACC GGGTATGACG GTCCCAGGAC AGAGGGTCGG TGAACAGGAA ATAGGTTCTC GAAGACCGAC

3901 GCTCTGATGA ATCCTATCGG CCCCTGCGCA GCCTCCTGGG CCACCCAGAG AATTCTGTGC GGGCACACAC TTATAGGCTC CTGGGACACT TGCTCCAACA
     CGAGACTACT TAGGATAGCC GGGGACGCGT CGGAGGACCC GGTGGGTCTC TTAAGACACG CCCGTGTGTG AATATCCGAG GACCCTGTGA ACGAGGTTGT

4001 CAGCATGGCC CTGCGTGGGG CACTGCAGAG CCAGTCTGGA CTGCTCAGCC TTCTGCTGCT TGGGCTTGGA GACAAGGATC CTGTTGTGCG GTGCAGTGCC
     GTCGTACCGG GACGCACCCC GTGACGTCTC GGTCAGACCT GACGAGTCGG AAGACGACGA ACCCGAACCT CTGTTCCTAG GACAACACGC CACGTCACGG

4101 AGCTTTGCTG TGGGCAATGC AGCCTACCAG GCTGGTCCTC TGGGACCTGC CCTGGCAGCT GCAGTGCCCA GTATGACCCA GCTGCTTGGA GATCCTCAGG
     TCGAAACGAC ACCCGTTACG TCGGATGGTC CGACCAGGAG ACCCTGGACG GGACCGTCGA CGTCACGGGT CATACTGGGT CGACGAACCT CTAGGAGTCC

4201 CTGGTATCCG GCGCAATGTT GCATCAGCTC TGGGCAACTT GGGACCTGAA GGTTTGGGAG AGGAGCTGTT ACAGTGCGAA GTACCCCAGC GGCTCCTAGA
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     TTACCGTACA CCTCTGGGGG TCGGTTTACA CTTCCTCCGA CGGGAGTAAC GGGAGGCCTC GGACGTTGTC CTCGGACCGT AGGTAGTCCA TGACCACAGG

4401 CTGGGTGCCA GTGAGAAACT ATCCTTGCTC TCTCTGGGGA ATCAGTCACT GCCACACAGC AGTCCTAGGC CTGCCTCTGC CAAACACTGC AGGAAACTCA
     GACCCACGGT CACTCTTTGA TAGGAACGAG AGAGACCCCT TAGTCAGTGA CGGTGTGTCG TCAGGATCCG GACGGAGACG GTTTGTGACG TCCTTTGAGT

4501 TTCACCTCCT GAGGCCAGCC CATAGCATGT GATTCCAGAT TCCTGCGGTC CAGCCTCCAA CTTTGGTTGC CAGCTCTTTC TTATTCTACT ACACAAGCCG
     AAGTGGAGGA CTCCGGTCGG GTATCGTACA CTAAGGTCTA AGGACGCCAG GTCGGAGGTT GAAACCAACG GTCGAGAAAG AATAAGATGA TGTGTTCGGC

4601 CCAACTCAAC TGAGAGCTAA AGAGACTAGA AAAGAGATAA GCTGCCAACT CAACTGAGAA CAAGAAACTA GAAGAGATTT ATATATAAAG CTTCTTCCTT
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FIG. 5E

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     TGAAGGAGAG GGAAGAACAG AAAGCCGAGG TTTTGGACGG AACACCTTAC CTCTTCGATC CGTCACTGCA ACGATGAGAG AAATGGGTAA GCGTACAGCA
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     GAGAGAACAC TCACGTCGTC GGACAGATAA CCCTGTCGAA CCAGTCGTTC CCCACTGGAA ACTGGAGGTC GGGTACCTTA CCTACCGACG TCGGTGTGTA
168   S  L  V   S  A  A  A   C  L  L   G  Q  L   G  Q  Q  G   V  T  F   D  L  Q   P  M  E  W   M  A  A   A  T  H

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     CGGAACAGAC GGGGACGTCT CGAGGAGTGA CTCCATGTCT ACCTAGAACC CTACCTACCC TTCATTTCTC TCTCCTTGAC CCGTAAAACC CCTCGGAGAC
201  A  L  S  A   P  A  E   L  L  T   E  V  Q  M   D  L  G   M  D  G   K  O

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     CTGGTCTCCT TACTTCTTCG TTGGGTGTCG GAAGGGAGAG TTCGATGACA CGGACACTAT CGGAACCTTG AAGGGGCGGA CGGGAGTCAT GACTGGGAAA

2901 GAAGGAAACC ATTCGCTGCG TCCCCTGGGA TCCAGTGGGA GATAAAATGA ATTCCCTGGG TTTCAGCAGA CATACACATG AGTTGTGAGG TCAGAGGGTT
     CTTCCTTTGG TAAGCGACGC AGGGGACCCT AGGTCACCCT CTATTTTACT TAAGGGACCC AAAGTCGTCT GTATGTGTAC TCAACACTCC AGTCTCCCAA

3001 AAGGTTTGAT AAGAAAATGA AATAAGACGA CAGGGAAATA CTAGGTGGGA AAGCGGAAGG AATTATTTCT GGGACTTCCT TTACTTGTAA GTCAGGGACA
     TTCCAAACTA TTCTTTTACT TTATTCTGCT GTCCCTTTAT GATCCACCCT TTCGCCTTCC TTAATAAAGA CCCTGAAGGA AATGAACATT CAGTCCCTGT

3101 GGAATGAATA AAAGCATTTG GATTCCTGAC TTCTGTCTTT CCCCCCGCCC TCTTTCACTT TTATCTCTAG CAGGGGAAGG CTAGCCTAAT CAGGGATATG
     CCTTACTTAT TTTCGTAAAC CTAAGGACTG AAGACAGAAA GGGGGGCGGG AGAAAGTGAA AATAGAGATC GTCCCCTTCC GATCGGATTA GTCCCTATAC

3201 TCCAGTTCAG AAATGTGGAC CGTTTTGTGG CACCGCTTCT CCATGGTCCT GAGGCTCCCC GAGGAGGCAT CTGCACAGGA AGGGGAGCTT TCGCTATCCA
     AGGTCAAGTC TTTACACCTG GCAAAACACC GTGGCGAAGA GGTACCAGGA CTCCGAGGGG CTCCTCCGTA GACGTGTCCT TCCCCTCGAA AGCGATAGGT

3301 GTCCACCAAG CCCTGAGCCA GACTGGACAC TGATTTCTCC CCAGGGCATG GCAGCCCTGC TGAGCCTGGC CATGGCCACC TTTACCCAGG AGCCCCAGTT
     CAGGTGGTTC GGGACTCGGT CTGACCTGTG ACTAAAGAGG GGTCCCGTAC CGTCGGGACG ACTCGGACCG GTACCGGTGG AAATGGGTCC TCGGGGTCAA

3401 ATGCCTGAGC TGCCTGTCCC AGCATGGAAG TATCCTCATG TCCATCCTGA AGCATCTGCT TTGCCCCAGC TTCCTGAATC AACTGCGCCA GGCGCCTCAT
     TACGGACTCG ACGGACAGGG TCGTACCTTC ATAGGAGTAC AGGTAGGACT TCGTAGACGA AACGGGGTCG AAGGACTTAG TTGACGCGGT CCGCGGAGTA

3501 GGGTCTGAGT TTCTCCCTGT CGTGGTGCTC TCTGTCTGCC AGCTCCTTTG CTTCCCCTTT GCGCTGGACA TGGATGCTGA CCTCCTTATA GGTGTCTTGG
     CCCAGACTCA AAGAGGGACA GCACCACGAG AGACAGACGG TCGAGGAAAC GAAGGGGAAA CGCGACCTGT ACCTACGACT GGAGGAATAT CCACAGAACC
```

FIG. 5D

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1601 GCCGGGAGGC AGGGCTTCCT GGGCTGCTGC TGAGTCTACT CAGGCACAGT CAGGAGAGCA ACAGCCTCCA GCAGCAATCT TGGTATGGGA CCTTCTTACA
     CGGCCCTCCG TCCCGAAGGA CCCGACGACG ACTCAGATGA GTCCGTGTCA GTCCTCTCGT TGTCGGAGGT CGTCGTTAGA ACCATACCCT GGAAGAATGT
 497  R  E  A   G  L  P   G  L  L  L   S  L  L   R  H  S   Q  E  S  N   S  L  Q   Q  Q  S   W  Y  G  T   F  L  Q

1701 GGACCTGATG GCTGTGATTC AGGCCTACTT TGCCTGTACC TTCAATCTGG AGAGGAGCCA GACAAGTGAC AGCCTGCAGG TGTTTCAGGA GGCTGCCAAC
     CCTGGACTAC CGACACTAAG TCCGGATGAA ACGGACATGG AAGTTAGACC TCTCCTCGGT CTGTTCACTG TCGGACGTCC ACAAAGTCCT CCGACGGTTG
 530  D  L  M   A  V  I  Q   A  Y  F   A  C  T   F  N  L  E   R  S  Q   T  S  D   S  L  Q  V   F  Q  E   A  A  N

1801 CTTTTTCTGG ACCTGTTGGG GAAACTGCTG GCCCAACCAG ATGACTCTGA GCAGACTTTG CGGAGGGACA GCCTTATGTG CTTTACTGTC CTGTGCGAAG
     GAAAAAGACC TGGACAACCC CTTTGACGAC CGGGTTGGTC TACTGAGACT CGTCTGAAAC GCCTCCCTGT CGGAATACAC GAAATGACAG GACACGCTTC
 563 L  F  L  D   L  L  G   K  L  L   A  Q  P  D   D  S  E   Q  T  L   R  R  D  S   L  M  C   F  T  V   L  C  E  A

1901 CCATGGATGG GAACAGCCGG GCCATCTCCA AAGCCTTTTA CTCCAGCTTG CTGACGACAC AGCAGGTTGT CTTGGATGGG CTCCTTCATG GCTTGACAGT
     GGTACCTACC CTTGTCGGCC CGGTAGAGGT TTCGGAAAAT GAGGTCGAAC GACTGCTGTG TCGTCCAACA GAACCTACCC GAGGAAGTAC CGAACTGTCA
 597  M  D  G   N  S  R   A  I  S  K   A  F  Y   S  S  L   L  T  T  Q   Q  V  V   L  D  G   L  L  H  G   L  T  V

2001 TCCACAGCTC CCTGTCCACA CTCCCCAAGG TTCCCTACTC CTGCTGCCAT GTCGGTGAGT ACTGGTGCTA TTGTCTAGGG CAAGAGCCTC AGGCCTTTGG
     AGGTGTCGAG GGACAGGTGT GAGGGGTTCC AAGGGATGAG GACGACGGTA CAGCCACTCA TGACCACGAT AACAGATCCC GTTCTCGGAG TCCGGAAACC
 630  P  Q  L   P  V  H  T   P  Q  G   S  L  L   L  L  P  C   R  O

2101 AGTTACTCTT TGCTTTTCTC CACAGGAGCC CCGCAAGTGA GCCAGCCACT GCGAGAGCAG AGTGAGGATA TACCTGGAGC CATTTCCTCT GCCCTGGCAG
     TCAATGAGAA ACGAAAAGAG GTGTCCTCGG GGCGTTCACT CGGTCGGTGA CGCTCTCGTC TCACTCCTAT ATGGACCTCG GTAAAGGAGA CGGGACCGTC
   1 S  Y  S  L   L  F  S   T  G  A   P  Q  V  S   Q  P  L   R  E  Q   S  E  D  I   P  G  A   I  S  S   A  L  A  A
     ^2nd ORF starts from here

2201 CCATATGCAC TGCTCCTGTG GGACTGCCCG ACTGCTGGGA TGCCAAGGAG CAGGTCTGTT GGCATTTGGC AAATCAGCTA ACTGAAGACA GCAGCCAGCT
     GGTATACGTG ACGAGGACAC CCTGACGGGC TGACGACCCT ACGGTTCCTC GTCCAGACAA CCGTAAACCG TTTAGTCGAT TGACTTCTGT CGTCGGTCGA
  35  I  C  T   A  P  V   G  L  P  D   C  W  D   A  K  E   Q  V  C  W   H  L  A   N  Q  L   T  E  D  S   S  Q  L

2301 CAGGCCATCC CTCATCTCTG GCCTGCAGCA TCCCATCCTG TGCCTGCACC TTCTCAAGGT TCTATACTCC TGCTGCCTTG TCAGTGAGGG CCTGTGCCGT
     GTCCGGTAGG GAGTAGAGAC CGGACGTCGT AGGGTAGGAC ACGGACGTGG AAGAGTTCCA AGATATGAGG ACGACGGAAC AGTCACTCCC GGACACGGCA
  68  R  P  S   L  I  S  G   L  Q  H   P  I  L   C  L  H  L   L  K  V   L  Y  S   C  C  L  V   S  E  G   L  C  R

2401 CTTCTGGGGC AGGAGCCCCT GGCCTTGGAA TCCCTGTTTA TGTTGATTCA GGGCAAGGTA AAAGTAGTAG ATTGGGAAGA GTCTACTGAA GTGACACTCT
     GAAGACCCCG TCCTCGGGGA CCGGAACCTT AGGGACAAAT ACAACTAAGT CCCGTTCCAT TTTCATCATC TAACCCTTCT CAGATGACTT CACTGTGAGA
 101 L  L  G  Q   E  P  L   A  L  E   S  L  F  M   L  I  Q   G  K  V   K  V  V  D   W  E  E   S  T  E   V  T  L  Y
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FIG. 5C

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701  GCACCCCTCC CTTCTATGCT ACAAGCATCT TTCAGCTGGT CAGCCTCATT CTCAAGGACC CTGTGCGCTG GCCCTCAACC ATCAGTCCCT GCTTTAAGAA
     CGTGGGGAGG GAAGATACGA TGTTCGTAGA AAGTCGACCA GTCGGAGTAA GAGTTCCTGG GACACGCGAC CGGGAGTTGG TAGTCAGGGA CGAAATTCTT
197   T  P  P   F  Y  A   T  S  I  F   Q  L  V   S  L  I   L  K  D  P   V  R  W   P  S  T   I  S  P  C   F  K  N

801  CTTCCTGCAG GGACTGCTCA CCAAAGACCC ACGGCAGCGA CTGTCCTGGC CAGACCTCTT ATATCACCCC TTTATTGCTG GTCATGTCAC CATAATAACT
     GAAGGACGTC CCTGACGAGT GGTTTCTGGG TGCCGTCGCT GACAGGACCG GTCTGGAGAA TATAGTGGGG AAATAACGAC CAGTACAGTG GTATTATTGA
230  F  L  Q   G  L  L  T   K  D  P   R  Q  R   L  S  W  P   D  L  L   Y  H  P   F  I  A  G   H  V  T   I  I  T

901  GAGCCAGCAG GCCCAGATTT GGGGACCCCA TTCACCAGCC GCCTACCCCC AGAACTTCAG GTCCTAAAGG ACGAACAGGC CCATCGGTTG GCCCCCAAGG
     CTCGGTCGTC CGGGTCTAAA CCCCTGGGGT AAGTGGTCGG CGGATGGGGG TCTTGAAGTC CAGGATTTCC TGCTTGTCCG GGTAGCCAAC CGGGGGTTCC
263  E  P  A  G   P  D  L   G  T  P   F  T  S  R   L  P  P   E  L  Q   V  L  K  D   E  Q  A   H  R  L   A  P  K  G

1001 GTAATCAGTC TCGCATCTTG ACTCAGGCCT ATAAACGCAT GGCTGAGGAG GCCATGCAGA AGAAACATCA GAACACAGGA CCTGCCCTTG AGCAAGAGGA
     CATTAGTCAG AGCGTAGAAC TGAGTCCGGA TATTTGCGTA CCGACTCCTC CGGTACGTCT TCTTTGTAGT CTTGTGTCCT GGACGGGAAC TCGTTCTCCT
297   N  Q  S   R  I  L   T  Q  A  Y   K  R  M   A  E  E   A  M  Q  K   K  H  Q   N  T  G   P  A  L  E   Q  E  D

1101 CAAGACCAGC AAGGTGGCTC CTGGCACAGC CCCTCTGCCC AGACTCGGGG CCACTCCTCA GGAATCAAGC CTCCTGGCCG GGATCTTAGC CTCAGAATTG
     GTTCTGGTCG TTCCACCGAG GACCGTGTCG GGGAGACGGG TCTGAGCCCC GGTGAGGAGT CCTTAGTTCG GAGGACCGGC CCTAGAATCG GAGTCTTAAC
330  K  T  S   K  V  A  P   G  T  A   P  L  P   R  L  G  A   T  P  Q   E  S  S   L  L  A  G   I  L  A   S  E  L

1201 AAGAGCAGCT GGGCTAAATC AGGGACTGGA GAGGTGCCCT CTGCACCTCG GGAAAACCGG ACCACCCCAG ATTGTGAACG AGCATTCCCA GAGGAGAGGC
     TTCTCGTCGA CCCGATTTAG TCCCTGACCT CTCCACGGGA GACGTGGAGC CCTTTTGGCC TGGTGGGGTC TAACACTTGC TCGTAAGGGT CTCCTCTCCG
363  K  S  S  W   A  K  S   G  T  G   E  V  P  S   A  P  R   E  N  R   T  T  P  D   C  E  R   A  F  P   E  E  R  P

1301 CAGAGGTGCT GGGCCAGCGG AGCACTGATG TAGTGGACCT GGAAAATGAG GAGCCAGACA GTGACAATGA GTGGCAGCAC CTGCTAGAGA CCACTGAGCC
     GTCTCCACGA CCCGGTCGCC TCGTGACTAC ATCACCTGGA CCTTTTACTC CTCGGTCTGT CACTGTTACT CACCGTCGTG GACGATCTCT GGTGACTCGG
397   E  V  L   G  Q  R   S  T  D  V   V  D  L   E  N  E   E  P  D  S   D  N  E   W  Q  H   L  L  E  T   T  E  P

1401 TGTGCCTATT CAACTGAAGG CTCCTCTCAC CTTGCTGTGT AATCCTGACT TCTGCCAGCG CATCCAGAGT CAGCTGCATG AAGCTGGAGG GCAGATCCTG
     ACACGGATAA GTTGACTTCC GAGGAGAGTG GAACGACACA TTAGGACTGA AGACGGTCGC GTAGGTCTCA GTCGACGTAC TTCGACCTCC CGTCTAGGAC
430  V  P  I   Q  L  K  A   P  L  T   L  L  C   N  P  D  F   C  Q  R   I  Q  S   Q  L  H  E   A  G  G   Q  I  L

1501 AAAGGCATCT TGGAGGGTGC TTCCCACATC CTGCCTGCAT TCCGGGTCCT GAGCAGTCTT CTCTCCAGCT GCAGTGATTC TGTTGCCTTG TATTCCTTCT
     TTTCCGTAGA ACCTCCCACG AAGGGTGTAG GACGGACGTA AGGCCCAGGA CTCGTCAGAA GAGAGGTCGA CGTCACTAAG ACAACGGAAC ATAAGGAAGA
463  K  G  I  L   E  G  A   S  H  I   L  P  A  F   R  V  L   S  S  L   L  S  S  C   S  D  S   V  A  L   Y  S  F  C
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FIG. 5B

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> length: 5252 bp (circular)

  1 GGAGCTTGGA GCTCCTAGGC TGGGGGCGTC CCAGATGTTG TGGAACTGTC CCTGGATCTA TAGCTCTTCA CCGTCTCTAC TTTCTTCCTT CTAAGAGATC
    CCTCGAACCT CGAGGATCCG ACCCCCGCAG GGTCTACAAC ACCTTGACAG GGACCTAGAT ATCGAGAAGT GGCAGAGATG AAAGAAGGAA GATTCTCTAG

101 CTGAAACCTC TGTCATGGAA AAGTACCACG TGTTGGAGAT GATTGGAGAA GGCTCTTTTG GGAGGGTGTA CAAGGGTCGA AGAAAATACA GTGCTCAGGT
    GACTTTGGAG ACAGTACCTT TTCATGGTGC ACAACCTCTA CTAACCTCTT CCGAGAAAAC CCTCCCACAT GTTCCCAGCT TCTTTTATGT CACGAGTCCA
  1               M  E   K  Y  H  V   L  E  M   I  G  E   G  S  F  G   R  V  Y   K  G  R   R  K  Y  S   A  Q  V
                ^Translation ATG starts here

201 CGTGGCCCTG AAGTTCATCC CAAAATTGGG GCGCTCAGAG AAGGAGCTGA GGAATTTGCA ACGAGAGATT GAAATAATGC GGGGTCTGCG GCATCCCAAC
    GCACCGGGAC TTCAAGTAGG GTTTTAACCC CGCGAGTCTC TTCCTCGACT CCTTAAACGT TGCTCTCTAA CTTTATTACG CCCCAGACGC CGTAGGGTTG
 30 V  A  L   K  F  I  P   K  L  G   R  S  E   K  E  L  R   N  L  Q   R  E  I   E  I  M  R   G  L  R   H  P  N

301 ATTGTGCATA TGCTTGACAG CTTTGAAACT GATAAAGAGG TGGTGGTGGT GACAGACTAT GCTGAGGGAG AGCTCTTTCA GATCCTAGAA GATGACGGAA
    TAACACGTAT ACGAACTGTC GAAACTTTGA CTATTTCTCC ACCACCACCA CTGTCTGATA CGACTCCCTC TCGAGAAAGT CTAGGATCTT CTACTGCCTT
 63 I  V  H  M   L  D  S   F  E  T   D  K  E  V   V  V  V   T  D  Y   A  E  G  E   L  F  Q   I  L  E   D  D  G  K

401 AACTTCCTGA AGACCAGGTT CAGGCCATTG CTGCCCAGTT GGTGTCAGCC CTGTACTATC TGCATTCCCA CCGCATCCTA CACCGAGATA TGAAGCCTCA
    TTGAAGGACT TCTGGTCCAA GTCCGGTAAC GACGGGTCAA CCACAGTCGG GACATGATAG ACGTAAGGGT GGCGTAGGAT GTGGCTCTAT ACTTCGGAGT
 97  L  P  E   D  Q  V   Q  A  I  A   A  Q  L   V  S  A   L  Y  Y  L   H  S  H   R  I  L   H  R  D  M   K  P  Q

501 GAACATCCTC CTCGCCAAGG GTGGTGGCAT CAAGCTCTGT GACTTTGGAT TTGCCCGGGC TATGAGCACC AATACAATGG TGCTGACATC CATCAAAGGC
    CTTGTAGGAG GAGCGGTTCC CACCACCGTA GTTCGAGACA CTGAAACCTA AACGGGCCCG ATACTCGTGG TTATGTTACC ACGACTGTAG GTAGTTTCCG
130  N  I  L   L  A  K  G   G  G  I   K  L  C   D  F  G  F   A  R  A   M  S  T   N  T  M  V   L  T  S   I  K  G

601 ACACCACTCT ATATGTCTCC AGAGCTGGTG GAGGAGCGAC CATACGACCA CACAGCGGAC CTCTGGTCTG TTGGCTGCAT ACTATATGAA CTGGCAGTAG
    TGTGGTGAGA TATACAGAGG TCTCGACCAC CTCCTCGCTG GTATGCTGGT GTGTCGCCTG GAGACCAGAC AACCGACGTA TGATATACTT GACCGTCATC
163 T  P  L  Y   M  S  P   E  L  V   E  E  R  P   Y  D  H   T  A  D   L  W  S  V   G  C  I   L  Y  E   L  A  V  G
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FIG. 5A

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4201 TCCATCAGGT ACTGGTGTCC CTGGGTGCCA GTGAGAAACT ATCCTTGCTC TCTCTGGGGA ATCAGTCACT GCCACACAGC AGTCCTAGGC CTGCCTCTGC
     AGGTAGTCCA TGACCACAGG GACCCACGGT CACTCTTTGA TAGGAACGAG AGAGACCCCT TAGTCAGTGA CGGTGTGTCG TCAGGATCCG GACGGAGACG
637   H  Q  V   L  V  S   L  G  A  S   E  K  L   S  L  L   S  L  G  N   Q  S  L   P  H  S   S  P  R  P   A  S  A

4301 CAAACACTGC AGGAAACTCA TTCACCTCCT GAGGCCAGCC CATAGCATGT GATTCCAGAT TCCTGCGGTC CAGCCTCCAA CTTTGGTTGC CAGCTCTTTC
     GTTTGTGACG TCCTTTGAGT AAGTGGAGGA CTCCGGTCGG GTATCGTACA CTAAGGTCTA AGGACGCCAG GTCGGAGGTT GAAACCAACG GTCGAGAAAG
670   K  H  C   R  K  L  I   H  L  L   R  P  A   H  S  M  O

4401 TTATTCTACT ACACAAGCCG CCAACTCAAC TGAGAGCTAA AGAGACTAGA AAAGAGATAA GCTGCCAACT CAACTGAGAA CAAGAAACTA GAAGAGATTT
     AATAAGATGA TGTGTTCGGC GGTTGAGTTG ACTCTCGATT TCTCTGATCT TTTCTCTATT CGACGGTTGA GTTGACTCTT GTTCTTTGAT CTTCTCTAAA

4501 ATATATAAAG CTTCTTCCTT CTCCCAGATG CAGGATGTTT TCAACCAGTA AATTTTATTG CTGTTGGTGC CAGAGAAGAG TCCTTTCTTC TCTACATCCA
     TATATATTTC GAAGAAGGAA GAGGGTCTAC GTCCTACAAA AGTTGGTCAT TTAAAATAAC GACAACCACG GTCTCTTCTC AGGAAAGAAG AGATGTAGGT

4601 GGGGCCTTTT CTCCAATAAT GTGCCTTTAA CTCTAGGGAC CTGCCTCACG GACCTTAGGG AAAAACCTCA ACCTGAAAGA TCTCTTCCTT TCTGGAGCTC
     CCCCGGAAAA GAGGTTATTA CACGGAAATT GAGATCCCTG GACGGAGTGC CTGGAATCCC TTTTTGGAGT TGGACTTTCT AGAGAAGGAA AGACCTCGAG

4701 CTTTAATCTT CCCAGCAGGT TTTTGCCTTA GACGTGCTGG CCCCAGGACA GTGATGAAGA CAGAGCCTGT CTCAGCTCTA GGCTGTGGGG ATCAATGCCA
     GAAATTAGAA GGGTCGTCCA AAAACGGAAT CTGCACGACC GGGGTCCTGT CACTACTTCT GTCTCGGACA GAGTCGAGAT CCGACACCCC TAGTTACGGT

4801 TCAGTCCCTG TTATTGAGGG ATTATCCCTT AGCCAACATT CCTATCTGTG GGTGGGCGTG GAGAGTGTAT CTTTTTTTGG GGTGTGTGTG TATATGTGTG
     AGTCAGGGAC AATAACTCCC TAATAGGGAA TCGGTTGTAA GGATAGACAC CCACCCGCAC CTCTCACATA GAAAAAAACC CCACACACAC ATATACACAC

4901 TGTGTATGTG TGTGTGTGTT TAATAGTTCT GTTTGTAAAC TCTTTTAATA AAAGTTGTGC CTCACCATAC TTGAAGCTCC CAGGACAAGG GTTGAGAGGC
     ACACATACAC ACACACACAA ATTATCAAGA CAAACATTTG AGAAAATTAT TTTCAACACG GAGTGGTATG AACTTCGAGG GTCCTGTTCC CAACTCTCCG

5001 TCAACCCCTC TTTCAGCTTC TATGTGGTGT TGGAGGTGCT GGTATCGTGT TCACACAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA
     AGTTGGGGAG AAAGTCGAAG ATACACCACA ACCTCCACGA CCATAGCACA AGTGTGTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT

5101 AAAAAAAAAA AAAAAAAAAA AAAAA
     TTTTTTTTTT TTTTTTTTTT TTTTT
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FIG. 4F

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3401 CCTCCTTATA GTTGTCTTGG CCGACCTCAG GGACTCAGAA GTTGCAGCCC ATCTGCTGCA GGTCTGCTGC TACCATCTTC CGTTGATGCA AGTGGAGCTG
     GGAGGAATAT CAACAGAACC GGCTGGAGTC CCTGAGTCTT CAACGTCGGG TAGACGACGT CCAGACGACG ATGGTAGAAG GCAACTACGT TCACCTCGAC
 370  L  L  I   V  V  L  A   D  L  R   D  S  E   V  A  A  H   L  L  Q   V  C  C   Y  H  L  P   L  M  Q   V  E  L

3501 CCCATCAGCC TTCTCACACG CCTGGCCCTC ATGGATCCCA CCTCTCTCAA CCAGTTTGTG AACACAGTGT CTGCCTCCCC TAGAACCATC GTCTCGTTTC
     GGGTAGTCGG AAGAGTGTGC GGACCGGGAG TACCTAGGGT GGAGAGAGTT GGTCAAACAC TTGTGTCACA GACGGAGGGG ATCTTGGTAG CAGAGCAAAG
 403 P  I  S  L   L  T  R   L  A  L   M  D  P  T   S  L  N   Q  F  V   N  T  V  S   A  S  P   R  T  I   V  S  F  L

3601 TCTCAGTTGC CCTCCTGAGT GACCAGCCAC TGTTGACCTC CGACCTTCTC TCTCTGCTGG CCCATACTGC CAGGGTCCTG TCTCCCAGCC ACTTGTCCTT
     AGAGTCAACG GGAGGACTCA CTGGTCGGTG ACAACTGGAG GCTGGAAGAG AGAGACGACC GGGTATGACG GTCCCAGGAC AGAGGGTCGG TGAACAGGAA
 437  S  V  A   L  L  S   D  Q  P  L   L  T  S   D  L  L   S  L  L  A   H  T  A   R  V  L   S  P  S  H   L  S  F

3701 TATCCAAGAG CTTCTGGCTG GCTCTGATGA ATCCTATCGG CCCCTGCGCA GCCTCCTGGG CCACCCAGAG AATTCTGTGC GGGCACACAC TTATAGGCTC
     ATAGGTTCTC GAAGACCGAC CGAGACTACT TAGGATAGCC GGGGACGCGT CGGAGGACCC GGTGGGTCTC TTAAGACACG CCCGTGTGTG AATATCCGAG
 470  I  Q  E   L  L  A  G   S  D  E   S  Y  R   P  L  R  S   L  L  G   H  P  E   N  S  V  R   A  H  T   Y  R  L

3801 CTGGGACACT TGCTCCAACA CAGCATGGCC CTGCGTGGGG CACTGCAGAG CCAGTCTGGA CTGCTCAGCC TTCTGCTGCT TGGGCTTGGA GACAAGGATC
     GACCCTGTGA ACGAGGTTGT GTCGTACCGG GACGCACCCC GTGACGTCTC GGTCAGACCT GACGAGTCGG AAGACGACGA ACCCGAACCT CTGTTCCTAG
 503 L  G  H  L   L  Q  H   S  M  A   L  R  G  A   L  Q  S   Q  S  G   L  L  S  L   L  L  L   G  L  G   D  K  D  P

3901 CTGTTGTGCG GTGCAGTGCC AGCTTTGCTG TGGGCAATGC AGCCTACCAG GCTGGTCCTC TGGGACCTGC CCTGGCAGCT GCAGTGCCCA GTATGACCCA
     GACAACACGC CACGTCACGG TCGAAACGAC ACCCGTTACG TCGGATGGTC CGACCAGGAG ACCCTGGACG GGACCGTCGA CGTCACGGGT CATACTGGGT
 537  V  V  R   C  S  A   S  F  A  V   G  N  A   A  Y  Q   A  G  P  L   G  P  A   L  A  A   A  V  P  S   M  T  Q

4001 GCTGCTTGGA GATCCTCAGG CTGGTATCCG GCGCAATGTT GCATCAGCTC TGGGCAACTT GGGACCTGAA GGTTTGGGAG AGGAGCTGTT ACAGTGCGAA
     CGACGAACCT CTAGGAGTCC GACCATAGGC CGCGTTACAA CGTAGTCGAG ACCCGTTGAA CCCTGGACTT CCAAACCCTC TCCTCGACAA TGTCACGCTT
 570  L  L  G   D  P  Q  A   G  I  R   R  N  V   A  S  A  L   G  N  L   G  P  E   G  L  G  E   E  L  L   Q  C  E

4101 GTACCCCAGC GGCTCCTAGA AATGGCATGT GGAGACCCCC AGCCAAATGT GAAGGAGGCT GCCCTCATTG CCCTCCGGAG CCTGCAACAG GAGCCTGGCA
     CATGGGGTCG CCGAGGATCT TTACCGTACA CCTCTGGGGG TCGGTTTACA CTTCCTCCGA CGGGAGTAAC GGGAGGCCTC GGACGTTGTC CTCGGACCGT
 603 V  P  Q  R   L  L  E   M  A  C   G  D  P  Q   P  N  V   K  E  A   A  L  I  A   L  R  S   L  Q  Q   E  P  G  I
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FIG. 4E

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2501 ATCCCTCATC TCTGGCCTGC AGCATCCCAT CCTGTGCCTG CACCTTCTCA AGGTTCTATA CTCCTGCTGC CTTGTCAGTG AGGGCCTGTG CCGTCTTCTG
     TAGGGAGTAG AGACCGGACG TCGTAGGGTA GGACACGGAC GTGGAAGAGT TCCAAGATAT GAGGACGACG GAACAGTCAC TCCCGGACAC GGCAGAAGAC
  70 S  L  I   S  G  L  Q   H  P  I   L  C  L   H  L  L  K   V  L  Y   S  C  C   L  V  S  E   G  L  C   R  L  L

2601 GGGCAGGAGC CCCTGGCCTT GGAATCCCTG TTTATGTTGA TTCAGGGCAA GGTAAAAGTA GTAGATTGGG AAGAGTCTAC TGAAGTGACA CTCTACTTCC
     CCCGTCCTCG GGGACCGGAA CCTTAGGGAC AAATACAACT AAGTCCCGTT CCATTTTCAT CATCTAACCC TTCTCAGATG ACTTCACTGT GAGATGAAGG
 103 G  Q  E  P   L  A  L   E  S  L   F  M  L  I   Q  G  K   V  K  V   V  D  W  E   E  S  T   E  V  T   L  Y  F  L

2701 TCTCCCTTCT TGTCTTTCGG CTCCAAAACC TGCCTTGTGG AATGGAGAAG CTAGGCAGTG ACGTTGCTAC TCTCTTTACC CATTCGCATG TCGTCTCTCT
     AGAGGGAAGA ACAGAAAGCC GAGGTTTTGG ACGGAACACC TTACCTCTTC GATCCGTCAC TGCAACGATG AGAGAAATGG GTAAGCGTAC AGCAGAGAGA
 137  S  L  L   V  F  R   L  Q  N  L   P  C  G   M  E  K   L  G  S  D   V  A  T   L  F  T   H  S  H  V   V  S  L

2801 TGTGAGTGCA GCAGCCTGTC TATTGGGACA GCTTGGTCAG CAAGGGGTGA CCTTTGACCT CCAGCCCATG GAATGGATGG CTGCAGCCAC ACATGCCTTG
     ACACTCACGT CGTCGGACAG ATAACCCTGT CGAACCAGTC GTTCCCCACT GGAAACTGGA GGTCGGGTAC CTTACCTACC GACGTCGGTG TGTACGGAAC
 170 V  S  A   A  A  C  L   L  G  Q   L  G  Q   Q  G  V  T   F  D  L   Q  P  M   E  W  M  A   A  A  T   H  A  L

2901 TCTGCCCCTG CAGAGGTTCG GTTGACTCCA CCAGGTAGTT GTGGATTCTA TGATGGCCTC CTTATCCTTC TGTTGCAGCT CCTCACTGAG CAGGGGAAGG
     AGACGGGGAC GTCTCCAAGC CAACTGAGGT GGTCCATCAA CACCTAAGAT ACTACCGGAG GAATAGGAAG ACAACGTCGA GGAGTGACTC GTCCCCTTCC
 203 S  A  P  A   E  V  R   L  T  P   P  G  S  C   G  F  Y   D  G  L   L  I  L  L   L  Q  L   L  T  E   Q  G  K  A

3001 CTAGCCTAAT CAGGGATATG TCCAGTTCAG AAATGTGGAC CGTTTTGTGG CACCGCTTCT CCATGGTCCT GAGGCTCCCC GAGGAGGCAT CTGCACAGGA
     GATCGGATTA GTCCCTATAC AGGTCAAGTC TTTACACCTG GCAAAACACC GTGGCGAAGA GGTACCAGGA CTCCGAGGGG CTCCTCCGTA GACGTGTCCT
 237  S  L  I   R  D  M   S  S  S  E   M  W  T   V  L  W   H  R  F  S   M  V  L   R  L  P   E  E  A  S   A  Q  E

3101 AGGGGAGCTT TCGCTATCCA GTCCACCAAG CCCTGAGCCA GACTGGACAC TGATTTCTCC CCAGGGCATG GCAGCCCTGC TGAGCCTGGC CATGGCCACC
     TCCCCTCGAA AGCGATAGGT CAGGTGGTTC GGGACTCGGT CTGACCTGTG ACTAAAGAGG GGTCCCGTAC CGTCGGGACG ACTCGGACCG GTACCGGTGG
 270  G  E  L   S  L  S  S   P  P  S   P  E  P   D  W  T  L   I  S  P   Q  G  M   A  A  L  L   S  L  A   M  A  T

3201 TTTACCCAGG AGCCCCAGTT ATGCCTGAGC TGCCTGTCCC AGCATGGAAG TATCCTCATG TCCATCCTGA AGCATCTGCT TTGCCCCAGC TTCCTGAATC
     AAATGGGTCC TCGGGGTCAA TACGGACTCG ACGGACAGGG TCGTACCTTC ATAGGAGTAC AGGTAGGACT TCGTAGACGA AACGGGGTCG AAGGACTTAG
 303 F  T  Q  E   P  Q  L   C  L  S   C  L  S  Q   H  G  S   I  L  M   S  I  L  K   H  L  L   C  P  S   F  L  N  Q

3301 AACTGCGCCA GGCGCCTCAT GGGTCTGAGT TTCTCCCTGT CGTGGTGCTC TCTGTCTGCC AGCTCCTTTG CTTCCCCTTT GCGCTGGACA TGGATGCTGA
     TTGACGCGGT CCGCGGAGTA CCCAGACTCA AAGAGGGACA GCACCACGAG AGACAGACGG TCGAGGAAAC GAAGGGGAAA CGCGACCTGT ACCTACGACT
 337  L  R  Q   A  P  H   G  S  E  F   L  P  V   V  V  L   S  V  C  Q   L  L  C   F  P  F   A  L  D  M   D  A  D
```

FIG. 4D

```
1601 TGCCGGGAGG CAGGGCTTCC TGGGCTGCTG CTGAGTCTAC TCAGGCACAG TCAGGAGAGC AACAGCCTCC AGCAGCAATC TTGGTATGGG ACCTTCTTAC
     ACGGCCCTCC GTCCCGAAGG ACCCGACGAC GACTCAGATG AGTCCGTGTC AGTCCTCTCG TTGTCGGAGG TCGTCGTTAG AACCATACCC TGGAAGAATG
 496 C R E A  G L P  G L L  L S L L  R H S  Q E S  N S L Q  Q Q S  W Y G  T F L Q

1701 AGGACCTGAT GGCTGTGATT CAGGCCTACT TTGCCTGTAC CTTCAATCTG GAGAGGAGCC AGACAAGTGA CAGCCTGCAG GTGTTTCAGG AGGCTGCCAA
     TCCTGGACTA CCGACACTAA GTCCGGATGA AACGGACATG GAAGTTAGAC CTCTCCTCGG TCTGTTCACT GTCGGACGTC CACAAAGTCC TCCGACGGTT
 530  D L M  A V I  Q A Y F  A C T  F N L  E R S Q  T S D  S L Q  V F Q E  A A N

1801 CCTTTTTCTG GACCTGTTGG GGAAACTGCT GGCCCAACCA GATGACTCTG AGCAGACTTT GCAGAGGGAC AGCCTTATGT GCTTTACTGT CCTGTGCGAA
     GGAAAAAGAC CTGGACAACC CCTTTGACGA CCGGGTTGGT CTACTGAGAC TCGTCTGAAA CGTCTCCCTG TCGGAATACA CGAAATGACA GGACACGCTT
 563 L F L  D L L G  K L L  A Q P  D D S E  Q T L  Q R D  S L M C  F T V  L C E

1901 GCCATGGATG GGAACAGCCG GGCCATCTCC AAAGCCTTTT ACTCCAGCTT GCTGACGACA CAGCAGGTTG TCTTGGATGG GCTCCTTCAT GGCTTGACAG
     CGGTACCTAC CCTTGTCGGC CCGGTAGAGG TTTCGGAAAA TGAGGTCGAA CGACTGCTGT GTCGTCCAAC AGAACCTACC CGAGGAAGTA CCGAACTGTC
 596 A M D G  N S R  A I S  K A F Y  S S L  L T T  Q Q V V  L D G  L L H  G L T V

2001 TTCCACAGCT CCCTGTCCAC ACTCCCCAAG GTAACCAGAG TGGAGAAGGG AGGTTCTCTT GACTTACTTG TTGCATAGGT CAGGCTCCGC TCTTTCTATT
     AAGGTGTCGA GGGACAGGTG TGAGGGGTTC CATTGGTCTC ACCTCTTCCC TCCAAGAGAA CTGAATGAAC AACGTATCCA GTCCGAGGCG AGAAAGATAA
 630  P Q L  P V H  T P Q G  N Q S  G E G  R F S O
                                                              ^Start of intron sequence

2101 GCCATCACCT AGATCGCACC TGGCATTTAG TAGGTGCTCA ATAAATAACT GTGAACTGAG AGAATGAATG GGGATCTGAG GGAAACAAAC AGACCTCATC
     CGGTAGTGGA TCTAGCGTGG ACCGTAAATC ATCCACGAGT TATTTATTGA CACTTGACTC TCTTACTTAC CCCTAGACTC CCTTTGTTTG TCTGGAGTAG

2201 CTGCATTCTT CCCACTCCCT TAGGTTCCCT ACTCCTGCTG CCATGTCGGT GAGTACTGGT GCTATTGTCT AGGGCAAGAG CCTCAGGCCT TTGGAGTTAC
     GACGTAAGAA GGGTGAGGGA ATCCAAGGGA TGAGGACGAC GGTACAGCCA CTCATGACCA CGATAACAGA TCCCGTTCTC GGAGTCCGGA AACCTCAATG
   1                                                                                                     S Y
                                                                            2nd ORF starts from here!^

2301 TCTTTGCTTT TCTCCACAGG AGCCCCGCAA GTGAGCCAGC CACTGCGAGA GCAGAGTGAG GATATACCTG GAGCCATTTC CTCTGCCCTG GCAGCCATAT
     AGAAACGAAA AGAGGTGTCC TCGGGGCGTT CACTCGGTCG GTGACGCTCT CGTCTCACTC CTATATGGAC CTCGGTAAAG GAGACGGGAC CGTCGGTATA
   3 S L L F  S T G  A P Q  V S Q P  L R E  Q S E  D I P G  A I S  S A L  A A I C

2401 GCACTGCTCC TGTGGGACTG CCCGACTGCT GGGATGCCAA GGAGCAGGTC TGTTGGCATT TGGCAAATCA GCTAACTGAA GACAGCAGCC AGCTCAGGCC
     CGTGACGAGG ACACCCTGAC GGGCTGACGA CCCTACGGTT CCTCGTCCAG ACAACCGTAA ACCGTTTAGT CGATTGACTT CTGTCGTCGG TCGAGTCCGG
  37  T A P  V G L  P D C W  D A K  E Q V  C W H L  A N Q  L T E  D S S Q  L R P
```

FIG. 4C

```
701 GGCACCCCTC CCTTCTATGC TACAAGCATC TTTCAGCTGG TCAGCCTCAT TCTCAAGGAC CCTGTGCGCT GGCCCTCAAC CATCAGTCCC TGCTTTAAGA
    CCGTGGGGAG GGAAGATACG ATGTTCGTAG AAAGTCGACC AGTCGGAGTA AGAGTTCCTG GGACACGCGA CCGGGAGTTG GTAGTCAGGG ACGAAATTCT
196 G  T  P  P   F  Y  A   T  S  I   F  Q  L  V   S  L  I   L  K  D   P  V  R  W   P  S  T   I  S  P   C  F  K  N

801 ACTTCCTGCA GGGACTGCTC ACCAAAGACC CACGGCAGCG ACTGTCCTGG CCAGACCTCT TATATCACCC CTTTATTGCT GGTCATGTCA CCATAATAAC
    TGAAGGACGT CCCTGACGAG TGGTTTCTGG GTGCCGTCGC TGACAGGACC GGTCTGGAGA ATATAGTGGG GAAATAACGA CCAGTACAGT GGTATTATTG
230  F  L  Q   G  L  L   T  K  D  P   R  Q  R   L  S  W   P  D  L  L   Y  H  P   F  I  A   G  H  V  T   I  I  T

901 TGAGCCAGCA GGCCCAGATT TGGGGACCCC ATTCACCAGC CGCCTACCCC CAGAACTTCA GGTCCTAAAG GACGAACAGG CCCATCGGTT GGCCCCCAAG
    ACTCGGTCGT CCGGGTCTAA ACCCCTGGGG TAAGTGGTCG GCGGATGGGG GTCTTGAAGT CCAGGATTTC CTGCTTGTCC GGGTAGCCAA CCGGGGGTTC
263  E  P  A   G  P  D  L   G  T  P   F  T  S   R  L  P  P   E  L  Q   V  L  K   D  E  Q  A   H  R  L   A  P  K

1001 GGTAATCAGT CTCGCATCTT GACTCAGGCC TATAAACGCA TGGCTGAGGA GGCCATGCAG AAGAAACATC AGAACACAGG ACCTGCCCTT GAGCAAGAGG
     CCATTAGTCA GAGCGTAGAA CTGAGTCCGG ATATTTGCGT ACCGACTCCT CCGGTACGTC TTCTTTGTAG TCTTGTGTCC TGGACGGGAA CTCGTTCTCC
296 G  N  Q  S   R  I  L   T  Q  A   Y  K  R  M   A  E  E   A  M  Q   K  K  H  Q   N  T  G   P  A  L   E  Q  E  D

1101 ACAAGACCAG CAAGGTGGCT CCTGGCACAG CCCCTCTGCC CAGACTCGGG GCCACTCCTC AGGAATCAAG CCTCCTGGCC GGGATCTTAG CCTCAGAATT
     TGTTCTGGTC GTTCCACCGA GGACCGTGTC GGGGAGACGG GTCTGAGCCC CGGTGAGGAG TCCTTAGTTC GGAGGACCGG CCCTAGAATC GGAGTCTTAA
330  K  T  S   K  V  A   P  G  T  A   P  L  P   R  L  G   A  T  P  Q   E  S  S   L  L  A   G  I  L  A   S  E  L

1201 GAAGAGCAGC TGGGCTAAAT CAGGGACTGG AGAGGTGCCC TCTGCACCTC GGGAAAACCG GACCACCCCA GATTGTGAAC GAGCATTCCC AGAGGAGAGG
     CTTCTCGTCG ACCCGATTTA GTCCCTGACC TCTCCACGGG AGACGTGGAG CCCTTTTGGC CTGGTGGGGT CTAACACTTG CTCGTAAGGG TCTCCTCTCC
363  K  S  S   W  A  K  S   G  T  G   E  V  P   S  A  P  R   E  N  R   T  T  P   D  C  E  R   A  F  P   E  E  R

1301 CCAGAGGTGC TGGGCCAGCG GAGCACTGAT GTAGTGGACC TGGAAAATGA GGAGCCAGAC AGTGACAATG AGTGGCAGCA CCTGCTAGAG ACCACTGAGC
     GGTCTCCACG ACCCGGTCGC CTCGTGACTA CATCACCTGG ACCTTTTACT CCTCGGTCTG TCACTGTTAC TCACCGTCGT GGACGATCTC TGGTGACTCG
396 P  E  V  L   G  Q  R   S  T  D   V  V  D  L   E  N  E   E  P  D   S  D  N  E   W  Q  H   L  L  E   T  T  E  P

1401 CTGTGCCTAT TCAACTGAAG GCTCCTCTCA CCTTGCTGTG TAATCCTGAC TTCTGCCAGC GCATCCAGAG TCAGCTGCAT GAAGCTGGAG GGCAGATCCT
     GACACGGATA AGTTGACTTC CGAGGAGAGT GGAACGACAC ATTAGGACTG AAGACGGTCG CGTAGGTCTC AGTCGACGTA CTTCGACCTC CCGTCTAGGA
430  V  P  I   Q  L  K   A  P  L  T   L  L  C.  N  P  D   F  C  Q  R   I  Q  S   Q  L  H   E  A  G  G   Q  I  L

1501 GAAAGGCATC TTGGAGGGTG CTTCCCACAT CCTGCCTGCA TTCCGGGTCC TGAGCAGTCT TCTCTCCAGC TGCAGTGATT CTGTTGCCTT GTATTCCTTC
     CTTTCCGTAG AACCTCCCAC GAAGGGTGTA GGACGGACGT AAGGCCCAGG ACTCGTCAGA AGAGAGGTCG ACGTCACTAA GACAACGGAA CATAAGGAAG
463  K  G  I   L  E  G  A   S  H  I   L  P  A   F  R  V  L   S  S  L   L  S  S   C  S  D  S   V  A  L   Y  S  F
```

FIG. 4B

```
> length: 5125 bp (circular)

  1 CCCACGCGTC CGCCCACGCG TCCGGGGCGT CCCAGATGTT GTGGAACTGT CCCTGGATCT ATAGCTCTTC ACCGTCTCTA CTTTCTTCCT TCTAAGAGAT
    GGGTGCGCAG GCGGGTGCGC AGGCCCCGCA GGGTCTACAA CACCTTGACA GGGACCTAGA TATCGAGAAG TGGCAGAGAT GAAAGAAGGA AGATTCTCTA

101 CCTGAAACCT CTGTCATGGA AAAGTACCAC GTGTTGGAGA TGATTGGAGA AGGCTCTTTT GGGAGGGTGT ACAAGGGTCG AAGAAAATAC AGTGCTCAGG
    GGACTTTGGA GACAGTACCT TTTCATGGTG CACAACCTCT ACTAACCTCT TCCGAGAAAA CCCTCCCACA TGTTCCCAGC TTCTTTTATG TCACGAGTCC
  1                M  E   K  Y  H   V  L  E  M   I  G  E   G  S  F   G  R  V  Y   K  G  R   R  K  Y   S  A  Q  V
                 ^Start of 1st ORF!

201 TCGTGGCCCT GAAGTTCATC CCAAAATTGG GGCGCTCAGA GAAGGAGCTG AGGAATTTGC AACGAGAGAT TGAAATAATG CGGGGTCTGC GGCATCCCAA
    AGCACCGGGA CTTCAAGTAG GGTTTTAACC CCGCGAGTCT CTTCCTCGAC TCCTTAAACG TTGCTCTCTA ACTTTATTAC GCCCCAGACG CCGTAGGGTT
 30  V  A  L   K  F  I   P  K  L  G   R  S  E   K  E  L   R  N  L  Q   R  E  I   E  I  M   R  G  L  R   H  P  N

301 CATTGTGCAT ATGCTTGACA GCTTTGAAAC TGATAAAGAG GTGGTGGTGG TGACAGACTA TGCTGAGGGA GAGCTCTTTC AGATCCTAGA AGATGACGGA
    GTAACACGTA TACGAACTGT CGAAACTTTG ACTATTTCTC CACCACCACC ACTGTCTGAT ACGACTCCCT CTCGAGAAAG TCTAGGATCT TCTACTGCCT
 63  I  V  H   M  L  D  S   F  E  T   D  K  E   V  V  V  V   T  D  Y   A  E  G   E  L  F  Q   I  L  E   D  D  G

401 AAACTTCCTG AAGACCAGGT TCAGGCCATT GCTGCCCAGT TGGTGTCAGC CCTGTACTAT CTGCATTCCC ACCGCATCCT ACACCGAGAT ATGAAGCCTC
    TTTGAAGGAC TTCTGGTCCA AGTCCGGTAA CGACGGGTCA ACCACAGTCG GGACATGATA GACGTAAGGG TGGCGTAGGA TGTGGCTCTA TACTTCGGAG
 96 K  L  P  E   D  Q  V   Q  A  I   A  A  Q  L   V  S  A   L  Y  Y   L  H  S  H   R  I  L   H  R  D   M  K  P  Q

501 AGAACATCCT CCTCGCCAAG GGTGGTGGCA TCAAGCTCTG TGACTTTGGA TTTGCCCGGG CTATGAGCAC CAATACAATG GTGCTGACAT CCATCAAAGG
    TCTTGTAGGA GGAGCGGTTC CCACCACCGT AGTTCGAGAC ACTGAAACCT AAACGGGCCC GATACTCGTG GTTATGTTAC CACGACTGTA GGTAGTTTCC
130  N  I  L   L  A  K   G  G  G  I   K  L  C   D  F  G   F  A  R  A   M  S  T   N  T  M   V  L  T  S   I  K  G

601 CACACCACTC TATATGTCTC CAGAGCTGGT GGAGGAGCGA CCATACGACC ACACAGCGGA CCTCTGGTCT GTTGGCTGCA TACTATATGA ACTGGCAGTA
    GTGTGGTGAG ATATACAGAG GTCTCGACCA CCTCCTCGCT GGTATGCTGG TGTGTCGCCT GGAGACCAGA CAACCGACGT ATGATATACT TGACCGTCAT
163  T  P  L   Y  M  S  P   E  L  V   E  E  R   P  Y  D  H   T  A  D   L  W  S   V  G  C  I   L  Y  E   L  A  V
```

FIG. 4A

hfused 1043 M D P T S L N Q F V N T V S A S P R T I V S F L S V A L L S D Q P L L T S D L L S L L A H T A R V L

hfused 1093 S P S H L S F I Q E L L A G S D E S Y R P L R S L L G H P E N S V R A H T Y R L L G H L L Q H S M A

hfused 1143 L R G A L Q S Q S G L L S L L L L G L G D K D P V V R C S A S F A V G N A A Y Q A G P L G P A L A A

hfused 1193 A V P S M T Q L L G D P Q A G I R R N V A S A L G N L G P E G L G E E L L Q C E V P Q R L L E M A C

hfused 1243 G D P Q P N V K E A A L I A L R S L Q Q E P G I H Q V L V S L G A S E K L S L L S L G N Q S L P H S

hfused 1293 S P R P A S A K H C R K L I H L L R P A H S M

FIG. 3E

```
hfused  744  M L I Q G K V K V V D W E E S T E V T L Y F L S L L V F R L Q N L P C G - M E K L G S D V A T L F T
dfused  720  V L L Q S R H H L L - R Q R A C Q M - - - L L L L A R F S L R G V Q C I W S G E L K S A L Q A W P M

hfused  793  H S H V V S L V S A A A C L L G Q L G Q Q G V T F D L Q P M E W M A A A T H A L S A P A E V R L T P
dfused  766  Q Q T C Q S L R L E A A Q T L D E L S Q F S F - F V A Q A T A

hfused  843  P G S C G F Y D G L L I L L L Q L L T E Q G K A S L I R D M S S S E M W T V L W H R F S M V L R L P

hfused  893  E E A S A Q E G E L S L S S P P S P E P D W T L I S P Q G M A A L L S L A M A T F T Q E P Q L C L S

hfused  943  C L S Q H G S I L M S I L K H L L C P S F L N Q L R Q A P H G S E F L P V V V L S V C Q L L C F P F

hfused  993  A L D M D A D L L I V V L A D L R D S E V A A H L L Q V C C Y H L P L M Q V E L P I S L L T R L A L
```

FIG. 3D

```
hfused  500  G L P G L L L S L L R - H S Q E S N S L Q Q Q S W Y G T F L Q D L M A V I Q A Y F A C T F N L E - R
dfused  487  S P P C L L P G W D S C D E S Q S P P I E N D E W L A F L H R S I Q E L L D G E F D S L K Q H N L V

hfused  548  S Q T S D S L Q V F Q E A A N L F L D L L G K L L A Q P D D S E Q T L Q R D S L M C F T V L C E A M
dfused  537  S I I V A P L R N S K A I P K V - L Q S V A Q L L S L P - - - - F V L A E Q H L V A E A I - - K G V

hfused  598  D G N S R A I S K A F Y S S L L T T Q Q V V L D G L L H G L T V P Q L P V H T P Q G A P Q V S Q P L
dfused  580  Y I D V K L V P N L M Y A C K L L L S Q R H L T D - - - - - S A A S L P A G T G V S L S R T V R S C

hfused  648  R E Q S E D I P G A I S S A L A A I C T A P V G L P D C W D A K E Q V C W H L A N - Q L T E D S S Q
dfused  625  S D L S A E E M S T A C S L Y E L V C H L V H Q Q Q Q F L - - - T Q F C D A V A I L A V N D M F I N

hfused  697  L R P S L I S G L Q H P I L C L H L L K V L Y S C C L V S E - - G L C R L L G Q E P L A L E - S L F
dfused  672  F L T H D F K D S R P V R L A S C M L - A L F - C C V L R E L P E N A E L V E K I V F D S R L Q L A
```

FIG. 3C

```
hfused  251  H P F I A G H V T I I T E P A G P D L G T P F T S R L P P E L Q V L K D E Q A H R L A P K G N Q S R
dfused  251  H P F V E G K L - Y I A E V Q A A Q T S P F I N P Q L A K D T K - - K S Q Q L R H V G A D L G D V -

hfused  301  I L T Q A Y K R M A E E A M Q K K H Q N T G P A L E Q E D K T S K V A P G T A P L P R L G A T P Q E
dfused  297  L A A L K L S D V A N E N L S T S R D S I N - A I A P S D I E Q L E T D V E D N V H R L - I V P - -

hfused  351  S S L L A G I L A S E L K S S W A K S G T G E V P S A P R E N R T T P D C - E R A F P E E R P E V L
dfused  343  - - - F A D I S Y R E L P C G - - - T A A A A R R A G A M P L I N S Q T C F V S G N S N M I L N H L

hfused  400  G Q R S T D V V D L E N E E P D S D N E W Q H L L E T T E P V P I Q L K A P L T L L C N P D F C Q R
dfused  387  N D N F A I E A P A S S A T K S M K S K L K L A L N I K Q S R S K D L E K R K L S Q N L D N F S L R

hfused  450  I Q S Q L H E A G G Q I L K G I L E G A S H I L P A F R V L S S L L S S C S D S V A L Y S F C R E A
dfused  437  L G Q S I D I E V Q R K T T E M L T Q Q S Q A Q Q L Q D R K T Q Q L K Q S M H S T N D E K L S S D N
```

FIG. 3B

hfused	1	MEKYHVLEMIGEGSFGRVYKGRRKYSAQVVALKFIPKLGRSEKELRNLQR
dfused	1	MDRYAVSSLVGQGSFGCVYKAQRRDDDKVVAIKVISKRGRSNRELKNLRR
hfused	51	EIEIMRGLRHPNIVHMLDSFETDKEVVVVTDYAEGELFQILEDDGKLPED
dfused	51	ECDIQARLKHPHVIEMVESFESKFDLFVVTEFALMDLHRYLSFNGAMPEE
hfused	101	QVQAIAAQLVSALYYLHSHRILHRDMKPQNILLAKGGGIKLCDFGFARAM
dfused	101	HAQRVVCHLVSALYYLHSNRILHRDLKPQNVLLDKNMHAKLCDFGLARNM
hfused	151	STNTMVLTSIKGTPLYMSPELVEERPYDHTADLWSVGCILYELAVGTPPF
dfused	151	TMGTHVLTSIKGTPLYMAPELLAEQPYDHQADMWSLGCIAYESMAGQPPF
hfused	201	YATSIFQLVSLILKDPVRWPSTISPCFKNFLQGLLTKDPRQRLSWPDLLY
dfused	201	CATSILHLVKLIKHEDVKWPSTLSSECRSFLQGLLEKDPSMRISWTQLLC

FIG. 3A

CCCGGGCTATGAGCACCAATACAATGGTGCTGACATCCATCAAAGGCACACCACTCTATA
TGTCTCCAGAGCTGGTGGAGGAGCGACCATACGACCACACAGCGGACCTCTGGTCTGTTG
GCTGCATACTATATGAACTGGCAGTAGGCACCCCTCCCTTCTAATGCTACAAGCATCTTT
CAGCTGGTCAGCC

FIG. 2

```
4101 GCATGTGATT CCAGATTCCT GCGGTCCAGC CTCCAACTTT GGTGCCAGCT CTTTCTTATN TAATACACAA GCGCCAAYTC AACTGAGAGC TAAAGAGACT
     CGTACACTAA GGTCTAAGGA CGCCAGGTCG GAGGTTGAAA CCACGGTCGA GAAAGAATAN ATTATGTGTT CGCGGTTRAG TTGACTCTCG ATTTCTCTGA
1315   M  O

4201 AGAAAAGAGA TAAGCTGCCA ACTCAACTGA GAACAGGAAA CTNGAAGAGA TTTATATATA AAGCTTCTTC CTTCTCCCAG ATGCAGGATG TTTTCAACCA
     TCTTTTCTCT ATTCGACGGT TGAGTTGACT CTTGTCCTTT GANCTTCTCT AAATATATAT TTCGAAGAAG GAAGAGGGTC TACGTCCTAC AAAAGTTGGT

4301 GTAAATTTTA TTGCTGTTGG TGCCAGAGAA GAGTCCCTTT CTTCTCTACA TCCAGGGGCC NTTTTCTCCA ATAATGTGCC TTTAACTCTA GGGACCTGCC
     CATTTAAAAT AACGACAACC ACGGTCTCTT CTCAGGGAAA GAAGAGATGT AGGTCCCCGG NAAAAGAGGT TATTACACGG AAATTGAGAT CCCTGGACGG

4401 TCACGGACCT TAGGGAAAAA CCTCAACCTG AAAGATCTCT TCCTTTCTGG AGCTCCTTTA ATCTTCCCAG CAGGTTTTTG CCTTAGACGT GCTGGCCCCA
     AGTGCCTGGA ATCCCTTTTT GGAGTTGGAC TTTCTAGAGA AGGAAAGACC TCGAGGAAAT TAGAAGGGTC GTCCAAAAAC GGAATCTGCA CGACCGGGGT

4501 GGACAGTGAT GAAGACAGAG CCTGTCTCAG CTCTAGGCTG TGGGGATCAA TGCCATCAGT CCCTGTTATT GAGGGATTAT CCCTTAGCCA ACATTCCTAT
     CCTGTCACTA CTTCTGTCTC GGACAGAGTC GAGATCCGAC ACCCCTAGTT ACGGTAGTCA GGGACAATAA CTCCCTAATA GGGAATCGGT TGTAAGGATA

4601 CTGTGGGTGG GCGTGGAGAG TGTATCTTTT TTTGGGGTGT GTGTGTATAT GTGTGTGTGT ATGTGTGTGT GTGTTTAATA GTTCTGTTTG TAAACTCTTT
     GACACCCACC CGCACCTCTC ACATAGAAAA AAACCCCACA CACACATATA CACACACACA TACACACACA CACAAATTAT CAAGACAAAC ATTTGAGAAA

4701 TAATAAAAGT TGTGCCTCAC CATACTTGAA GCTCCCAGGA CAAGGGTTGA GAGGCTCAAC CCCTCTTTCA GCTTCTATGT GGTGTTGGAG GTGCTGGTAT
     ATTATTTTCA ACACGGAGTG GTATGAACTT CGAGGGTCCT GTTCCCAACT CTCCGAGTTG GGGAGAAAGT CGAAGATACA CCACAACCTC CACGACCATA

4801 CGTGTTCACA CAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA
     GCACAAGTGT GTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT TTTTTTTTTT
```

FIG. 1F

```
3501 TGCGCAGCCT CCTGGGCCAC CCAGAGAATT CTGTGCGGGC ACACACTTAT AGGCTCCTGG GACACTTGCT CCAACACAGC ATGGCCCTGC GTGGGGCACT
     ACGCGTCGGA GGACCCGGTG GGTCTCTTAA GACACGCCCG TGTGTGAATA TCCGAGGACC CTGTGAACGA GGTTGTGTCG TACCGGGACG CACCCCGTGA
1115  R  S  L   L  G  H   P  E  N   S   V  R  A   H  T  Y   R  L  L  G   H  L  L   Q  H  S   M  A  L  R   G  A  L

3601 GCAGAGCCAG TCTGGACTGC TCAGCCTTCT GCTGCTTGGG CTTGGAGACA AGGATCCTGT TGTGCGGTGC AGTGCCAGCT TTGCTGTGGG CAATGCAGCC
     CGTCTCGGTC AGACCTGACG AGTCGGAAGA CGACGAACCC GAACCTCTGT TCCTAGGACA ACACGCCACG TCACGGTCGA AACGACACCC GTTACGTCGG
1148 Q  S  Q   S  G  L  L   S  L  L   L  L  G   L  G  D  K   D  P  V   V  R  C   S  A  S  F   A  V  G   N  A  A

3701 TACCAGGCTG GTCCTCTGGG ACCTGCCCTG GCAGCTGCAG TGCCCAGTAT GACCCAGCTG CTTGGAGATC CTCAGGCTGG TATCCGGCGC AATGTTGCAT
     ATGGTCCGAC CAGGAGACCC TGGACGGGAC CGTCGACGTC ACGGGTCATA CTGGGTCGAC GAACCTCTAG GAGTCCGACC ATAGGCCGCG TTACAACGTA
1181 Y  Q  A  G   P  L  G   P  A  L   A  A  A  V   P  S  M   T  Q  L   L  G  D  P   Q  A  G   I  R  R   N  V  A  S

3801 CAGCTCTGGG CAACTTGGGA CCTGAAGGTT TGGGAGAGGA GCTGTTACAG TGCGAAGTAC CCCAGCGGCT CCTAGAAATG GCATGTGGAG ACCCCCAGCC
     GTCGAGACCC GTTGAACCCT GGACTTCCAA ACCCTCTCCT CGACAATGTC ACGCTTCATG GGGTCGCCGA GGATCTTTAC CGTACACCTC TGGGGGTCGG
1215  A  L  G   N  L  G   P  E  G  L   G  E  E   L  L  Q   C  E  V  P   Q  R  L   L  E  M   A  C  G  D   P  Q  P

3901 AAATGTGAAG GAGGCTGCCC TCATTGCCCT CCGGAGCCTG CAACAGGAGC CTGGCATCCA TCAGGTACTG GTGTCCCTGG GTGCCAGTGA GAAACTATCC
     TTTACACTTC CTCCGACGGG AGTAACGGGA GGCCTCGGAC GTTGTCCTCG GACCGTAGGT AGTCCATGAC CACAGGGACC CACGGTCACT CTTTGATAGG
1248  N  V  K   E  A  A  L   I  A  L   R  S  L   Q  Q  E  P   G  I  H   Q  V  L   V  S  L  G   A  S  E   K  L  S

4001 TTGCTCTCTC TGGGGAATCA GTCACTGCCA CACAGCAGTC CTAGGCCTGC CTCTGCCAAA CACTGCAGGA AACTCATTCA CCTCCTGAGG CCAGCCCATA
     AACGAGAGAG ACCCCTTAGT CAGTGACGGT GTGTCGTCAG GATCCGGACG GAGACGGTTT GTGACGTCCT TTGAGTAAGT GGAGGACTCC GGTCGGGTAT
1281 L  L  S  L   G  N  Q   S  L  P   H  S  S  P   R  P  A   S  A  K   H  C  R  K   L  I  H   L  L  R   P  A  H  S
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FIG. 1E

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2601 GGGTGACCTT TGACCTCCAG CCCATGGAAT GGATGGCTGC AGCCACACAT GCCTTGTCTG CCCCTGCAGA GGTTCGGTTG ACTCCACCAG GTAGTTGTGG
     CCCACTGGAA ACTGGAGGTC GGGTACCTTA CCTACCGACG TCGGTGTGTA CGGAACAGAC GGGGACGTCT CCAAGCCAAC TGAGGTGGTC CATCAACACC
815   V  T  F   D  L  Q   P  M  E  W   M  A  A   A  T  H   A  L  S  A   P  A  E   V  R  L   T  P  P  G   S  C  G

2701 ATTCTATGAT GGCCTCCTTA TCCTTCTGTT GCAGCTCCTC ACTGAGCAGG GGAAGGCTAG CCTAATCAGG GATATGTCCA GTTCAGAAAT GTGGACCGTT
     TAAGATACTA CCGGAGGAAT AGGAAGACAA CGTCGAGGAG TGACTCGTCC CCTTCCGATC GGATTAGTCC CTATACAGGT CAAGTCTTTA CACCTGGCAA
848  F  Y  D   G  L  L  I   L  L  L   Q  L  L   T  E  Q  G   K  A  S   L  I  R   D  M  S  S   S  E  M   W  T  V

2801 TTGTGGCACC GCTTCTCCAT GGTCCTGAGG CTCCCCGAGG AGGCATCTGC ACAGGAAGGG GAGCTTTCGC TATCCAGTCC ACCAAGCCCT GAGCCAGACT
     AACACCGTGG CGAAGAGGTA CCAGGACTCC GAGGGGCTCC TCCGTAGACG TGTCCTTCCC CTCGAAAGCG ATAGGTCAGG TGGTTCGGGA CTCGGTCTGA
881 L  W  H  R   F  S  M   V  L  R   L  P  E  E   A  S  A   Q  E  G   E  L  S  L   S  S  P   P  S  P   E  P  D  W

2901 GGACACTGAT TTCTCCCCAG GGCATGGCAG CCCTGCTGAG CCTGGCCATG GCCACCTTTA CCCAGGAGCC CCAGTTATGC CTGAGCTGCC TGTCCCAGCA
     CCTGTGACTA AAGAGGGGTC CCGTACCGTC GGGACGACTC GGACCGGTAC CGGTGGAAAT GGGTCCTCGG GGTCAATACG GACTCGACGG ACAGGGTCGT
915   T  L  I   S  P  Q   G  M  A  A   L  L  S   L  A  M   A  T  F  T   Q  E  P   Q  L  C   L  S  C  L   S  Q  H

3001 TGGAAGTATC CTCATGTCCA TCCTGAAGCA TCTGCTTTGC CCCAGCTTCC TGAATCAACT GCGCCAGGCG CCTCATGGGT CTGAGTTTCT CCCTGTCGTG
     ACCTTCATAG GAGTACAGGT AGGACTTCGT AGACGAAACG GGGTCGAAGG ACTTAGTTGA CGCGGTCCGC GGAGTACCCA GACTCAAAGA GGGACAGCAC
948   G  S  I   L  M  S  I   L  K  H   L  L  C   P  S  F  L   N  Q  L   R  Q  A   P  H  G  S   E  F  L   P  V  V

3101 GTGCTCTCTG TCTGCCAGCT CCTTTGCTTC CCCTTTGCGC TGGACATGGA TGCTGACCTC CTTATAGTTG TCTTGGCCGA CCTCAGGGAC TCAGAAGTTG
     CACGAGAGAC AGACGGTCGA GGAAACGAAG GGGAAACGCG ACCTGTACCT ACGACTGGAG GAATATCAAC AGAACCGGCT GGAGTCCCTG AGTCTTCAAC
981 V  L  S  V   C  Q  L   L  C  F   P  F  A  L   D  M  D   A  D  L   L  I  V  V   L  A  D   L  R  D   S  E  V  A

3201 CAGCCCATCT GCTGCAGGTC TGCTGCTACC ATCTTCCGTT GATGCAAGTG GAGCTGCCCA TCAGCCTTCT CACACGCCTG GCCCTCATGG ATCCCACCTC
     GTCGGGTAGA CGACGTCCAG ACGACGATGG TAGAAGGCAA CTACGTTCAC CTCGACGGGT AGTCGGAAGA GTGTGCGGAC CGGGAGTACC TAGGGTGGAG
1015  A  H  L   L  Q  V   C  C  Y  H   L  P  L   M  Q  V   E  L  P  I   S  L  L   T  R  L   A  L  M  D   P  T  S

3301 TCTCAACCAG TTTGTGAACA CAGTGTCTGC CTCCCCTAGA ACCATCGTCT CGTTTCTCTC AGTTGCCCTC CTGAGTGACC AGCCACTGTT GACCTCCGAC
     AGAGTTGGTC AAACACTTGT GTCACAGACG GAGGGGATCT TGGTAGCAGA GCAAAGAGAG TCAACGGGAG GACTCACTGG TCGGTGACAA CTGGAGGCTG
1048 L  N  Q   F  V  N  T   V  S  A   S  P  R   T  I  V  S   F  L  S   V  A  L   L  S  D  Q   P  L  L   T  S  D

3401 CTTCTCTCTC TGCTGGCCCA TACTGCCAGG GTCCTGTCTC CCAGCCACTT GTCCTTTATC CAAGAGCTTC TGGCTGGCTC TGATGAATCC TATCGGCCCC
     GAAGAGAGAG ACGACCGGGT ATGACGGTCC CAGGACAGAG GGTCGGTGAA CAGGAAATAG GTTCTCGAAG ACCGACCGAG ACTACTTAGG ATAGCCGGGG
1081 L  L  S  L   L  A  H   T  A  R   V  L  S  P   S  H  L   S  F  I   Q  E  L  L   A  G  S   D  E  S   Y  R  P  L
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FIG. 1D

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1701 AGAGCAACAG CCTCCAGCAG CAATCTTGGT ATGGGACCTT CTTACAGGAC CTGATGGCTG TGATTCAGGC CTACTTTGCC TGTACCTTCA ATCTGGAGAG
     TCTCGTTGTC GGAGGTCGTC GTTAGAACCA TACCCTGGAA GAATGTCCTG GACTACCGAC ACTAAGTCCG GATGAAACGG ACATGGAAGT TAGACCTCTC
515   S  N  S   L  Q  Q   Q  S  W  Y   G  T  F   L  Q  D   L  M  A  V   I  Q  A   Y  F  A   C  T  F  N   L  E  R

1801 GAGCCAGACA AGTGACAGCC TGCAGGTGTT TCAGGAGGCT GCCAACCTTT TTCTGGACCT GTTGGGGAAA CTGCTGGCCC AACCAGATGA CTCTGAGCAG
     CTCGGTCTGT TCACTGTCGG ACGTCCACAA AGTCCTCCGA CGGTTGGAAA AAGACCTGGA CAACCCCTTT GACGACCGGG TTGGTCTACT GAGACTCGTC
548   S  Q  T   S  D  S  L   Q  V  F   Q  E  A   A  N  L  F   L  D  L   L  G  K   L  L  A  Q   P  D  D   S  E  Q

1901 ACTTTGCGGA GGGACAGCCT TATGTGCTTT ACTGTCCTGT GCGAAGCCAT GGATGGGAAC AGCCGGGCCA TCTCCAAAGC CTTTTACTCC AGCTTGCTGA
     TGAAACGCCT CCCTGTCGGA ATACACGAAA TGACAGGACA CGCTTCGGTA CCTACCCTTG TCGGCCCGGT AGAGGTTTCG GAAAATGAGG TCGAACGACT
581  T  L  R  R   D  S  L   M  C  F   T  V  L  C   E  A  M   D  G  N   S  R  A  I   S  K  A   F  Y  S   S  L  L  T

2001 CGACACAGCA GGTTGTCTTG GATGGGCTCC TTCATGGCTT GACAGTTCCA CAGCTCCCTG TCCACACTCC CCAAGGAGCC CCGCAAGTGA GCCAGCCACT
     GCTGTGTCGT CCAACAGAAC CTACCCGAGG AAGTACCGAA CTGTCAAGGT GTCGAGGGAC AGGTGTGAGG GGTTCCTCGG GGCGTTCACT CGGTCGGTGA
615   T  Q  Q   V  V  L   D  G  L  L   H  G  L   T  V  P   Q  L  P  V   H  T  P   Q  G  A   P  Q  V  S   Q  P  L

2101 GCGAGAGCAG AGTGAGGATA TACCTGGAGC CATTTCCTCT GCCCTGGCAG CCATATGCAC TGCTCCTGTG GGACTGCCCG ACTGCTGGGA TGCCAAGGAG
     CGCTCTCGTC TCACTCCTAT ATGGACCTCG GTAAAGGAGA CGGGACCGTC GGTATACGTG ACGAGGACAC CCTGACGGGC TGACGACCCT ACGGTTCCTC
648   R  E  Q   S  E  D  I   P  G  A   I  S  S   A  L  A  A   I  C  T   A  P  V   G  L  P  D   C  W  D   A  K  E

2201 CAGGTCTGTT GGCATTTGGC AAATCAGCTA ACTGAAGACA GCAGCCAGCT CAGGCCATCC CTCATCTCTG GCCTGCAGCA TCCCATCCTG TGCCTGCACC
     GTCCAGACAA CCGTAAACCG TTTAGTCGAT TGACTTCTGT CGTCGGTCGA GTCCGGTAGG GAGTAGAGAC CGGACGTCGT AGGGTAGGAC ACGGACGTGG
681  Q  V  C  W   H  L  A   N  Q  L   T  E  D  S   S  Q  L   R  P  S   L  I  S  G   L  Q  H   P  I  L   C  L  H  L

2301 TTCTCAAGGT TCTATACTCC TGCTGCCTTG TCAGTGAGGG CCTGTGCCGT CTTCTGGGGC AGGAGCCCCT GGCCTTGGAA TCCCTGTTTA TGTTGATTCA
     AAGAGTTCCA AGATATGAGG ACGACGGAAC AGTCACTCCC GGACACGGCA GAAGACCCCG TCCTCGGGGA CCGGAACCTT AGGGACAAAT ACAACTAAGT
715   L  K  V   L  Y  S   C  C  L  V   S  E  G   L  C  R   L  L  G  Q   E  P  L   A  L  E   S  L  F  M   L  I  Q

2401 GGGCAAGGTA AAAGTAGTAG ATTGGGAAGA GTCTACTGAA GTGACACTCT ACTTCCTCTC CCTTCTTGTC TTTCGGCTCC AAAACCTGCC TTGTGGAATG
     CCCGTTCCAT TTTCATCATC TAACCCTTCT CAGATGACTT CACTGTGAGA TGAAGGAGAG GGAAGAACAG AAAGCCGAGG TTTTGGACGG AACACCTTAC
748   G  K  V   K  V  V  D   W  E  E   S  T  E   V  T  L  Y   F  L  S   L  L  V   F  R  L  Q   N  L  P   C  G  M

2501 GAGAAGCTAG GCAGTGACGT TGCTACTCTC TTTACCCATT CGCATGTCGT CTCTCTTGTG AGTGCAGCAG CCTGTCTATT GGGACAGCTT GGTCAGCAAG
     CTCTTCGATC CGTCACTGCA ACGATGAGAG AAATGGGTAA GCGTACAGCA GAGAGAACAC TCACGTCGTC GGACAGATAA CCCTGTCGAA CCAGTCGTTC
781  E  K  L  G   S  D  V   A  T  L   F  T  H  S   H  V  V   S  L  V   S  A  A  A   C  L  L   G  Q  L   G  Q  Q  G
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FIG. 1C

2 / 31

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 801 AGGACCCTGT GCGCTGGCCC TCAACCATCA GTCCCTGCTT TAAGAACTTC CTGCAGGGAC TGCTCACCAA AGACCCACGG CAGCGACTGT CCTGGCCAGA
     TCCTGGGACA CGCGACCGGG AGTTGGTAGT CAGGGACGAA ATTCTTGAAG GACGTCCCTG ACGAGTGGTT TCTGGGTGCC GTCGCTGACA GGACCGGTCT
 215  D  P  V   R  W  P   S  T  I  S   P  C  F   K  N  F   L  Q  G  L   L  T  K   D  P  R   Q  R  L  S   W  P  D

 901 CCTCTTATAT CACCCCTTTA TTGCTGGTCA TGTCACCATA ATAACTGAGC CAGCAGGCCC AGATTTGGGG ACCCCATTCA CCAGCCGCCT ACCCCCAGAA
     GGAGAATATA GTGGGGAAAT AACGACCAGT ACAGTGGTAT TATTGACTCG GTCGTCCGGG TCTAAACCCC TGGGGTAAGT GGTCGGCGGA TGGGGGTCTT
 248 L  L  Y   H  P  F  I   A  G  H   V  T  I   I  T  E  P   A  G  P   D  L  G   T  P  F  T   S  R  L   P  P  E

1001 CTTCAGGTCC TAAAGGACGA ACAGGCCCAT CGGTTGGCCC CCAAGGGTAA TCAGTCTCGC ATCTTGACTC AGGCCTATAA ACGCATGGCT GAGGAGGCCA
     GAAGTCCAGG ATTTCCTGCT TGTCCGGGTA GCCAACCGGG GGTTCCCATT AGTCAGAGCG TAGAACTGAG TCCGGATATT TGCGTACCGA CTCCTCCGGT
 281 L  Q  V  L   K  D  E   Q  A  H   R  L  A  P   K  G  N   Q  S  R   I  L  T  Q   A  Y  K   R  M  A   E  E  A  M

1101 TGCAGAAGAA ACATCAGAAC ACAGGACCTG CCCTTGAGCA AGAGGACAAG ACCAGCAAGG TGGCTCCTGG CACAGCCCCT CTGCCCAGAC TCGGGGCCAC
     ACGTCTTCTT TGTAGTCTTG TGTCCTGGAC GGGAACTCGT TCTCCTGTTC TGGTCGTTCC ACCGAGGACC GTGTCGGGGA GACGGGTCTG AGCCCCGGTG
 315  Q  K  K   H  Q  N   T  G  P  A   L  E  Q   E  D  K   T  S  K  V   A  P  G   T  A  P   L  P  R  L   G  A  T

1201 TCCTCAGGAA TCAAGCCTCC TGGCCGGGAT CTTAGCCTCA GAATTGAAGA GCAGCTGGGC TAAATCAGGG ACTGGAGAGG TGCCCTCTGC ACCTCGGGAA
     AGGAGTCCTT AGTTCGGAGG ACCGGCCCTA GAATCGGAGT CTTAACTTCT CGTCGACCCG ATTTAGTCCC TGACCTCTCC ACGGGAGACG TGGAGCCCTT
 348  P  Q  E   S  S  L  L   A  G  I   L  A  S   E  L  K  S   S  W  A   K  S  G   T  G  E  V   P  S  A   P  R  E

1301 AACCGGACCA CCCCAGATTG TGAACGAGCA TTCCCAGAGG AGAGGCCAGA GGTGCTGGGC CAGCGGAGCA CTGATGTAGT GGACCTGGAA AATGAGGAGC
     TTGGCCTGGT GGGGTCTAAC ACTTGCTCGT AAGGGTCTCC TCTCCGGTCT CCACGACCCG GTCGCCTCGT GACTACATCA CCTGGACCTT TTACTCCTCG
 381 N  R  T  T   P  D  C   E  R  A   F  P  E  E   R  P  E   V  L  G   Q  R  S  T   D  V  V   D  L  E   N  E  E  P

1401 CAGACAGTGA CAATGAGTGG CAGCACCTGC TAGAGACCAC TGAGCCTGTG CCTATTCAAC TGAAGGCTCC TCTCACCTTG CTGTGTAATC CTGACTTCTG
     GTCTGTCACT GTTACTCACC GTCGTGGACG ATCTCTGGTG ACTCGGACAC GGATAAGTTG ACTTCCGAGG AGAGTGGAAC GACACATTAG GACTGAAGAC
 415  D  S  D   N  E  W   Q  H  L  L   E  T  T   E  P  V   P  I  Q  L   K  A  P   L  T  L   L  C  N  P   D  F  C

1501 CCAGCGCATC CAGAGTCAGC TGCATGAAGC TGGAGGGCAG ATCCTGAAAG GCATCTTGGA GGGTGCTTCC CACATCCTGC CTGCATTCCG GGTCCTGAGC
     GGTCGCGTAG GTCTCAGTCG ACGTACTTCG ACCTCCCGTC TAGGACTTTC CGTAGAACCT CCCACGAAGG GTGTAGGACG GACGTAAGGC CCAGGACTCG
 448  Q  R  I   Q  S  Q  L   H  E  A   G  G  Q   I  L  K  G   I  L  E   G  A  S   H  I  L  P   A  F  R   V  L  S

1601 AGTCTTCTCT CCAGCTGCAG TGATTCTGTT GCCTTGTATT CCTTCTGCCG GGAGGCAGGG CTTCCTGGGC TGCTGCTGAG TCTACTCAGG CACAGTCAGG
     TCAGAAGAGA GGTCGACGTC ACTAAGACAA CGGAACATAA GGAAGACGGC CCTCCGTCCC GAAGGACCCG ACGACGACTC AGATGAGTCC GTGTCAGTCC
 481 S  L  L  S   S  C  S   D  S  V   A  L  Y  S   F  C  R   E  A  G   L  P  G  L   L  L  S   L  L  R   H  S  Q  E
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FIG. 1B

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> length: 4880 bp (circular)

  1 CCCGGGGATC CTCTAGAGAT CCCTCGACCT CGACCCACGC GTCCGCCCAC GCGTCCGCCC ACGCGTCCGG GGCGTCCCAG ATGTTGTGGA ACTGTCCCTG
    GGGCCCCTAG GAGATCTCTA GGGAGCTGGA GCTGGGTGCG CAGGCGGGTG CGCAGGCGGG TGCGCAGGCC CCGCAGGGTC TACAACACCT TGACAGGGAC

101 GATCTATAGC TCTTCACCGT CTCTACTTTC TTCCTTCTAA GAGATCCTGA AACCTCTGTC ATGGAAAAGT ACCACGTGTT GGAGATGATT GGAGAAGGCT
    CTAGATATCG AGAAGTGGCA GAGATGAAAG AAGGAAGATT CTCTAGGACT TTGGAGACAG TACCTTTTCA TGGTGCACAA CCTCTACTAA CCTCTTCCGA
  1                                                                M  E  K  Y   H  V  L   E  M  I   G  E  G  S

201 CTTTTGGGAG GGTGTACAAG GGTCGAAGAA AATACAGTGC TCAGGTCGTG GCCCTGAAGT TCATCCCAAA ATTGGGGCGC TCAGAGAAGG AGCTGAGGAA
    GAAAACCCTC CCACATGTTC CCAGCTTCTT TTATGTCACG AGTCCAGCAC CGGGACTTCA AGTAGGGTTT TAACCCCGCG AGTCTCTTCC TCGACTCCTT
 15   F  G  R   V  Y  K   G  R  R  K   Y  S  A   Q  V  V   A  L  K  F   I  P  K   L  G  R   S  E  K  E   L  R  N

301 TTTGCAACGA GAGATTGAAA TAATGCGGGG TCTGCGGCAT CCCAACATTG TGCATATGCT TGACAGCTTT GAAACTGATA AAGAGGTGGT GGTGGTGACA
    AAACGTTGCT CTCTAACTTT ATTACGCCCC AGACGCCGTA GGGTTGTAAC ACGTATACGA ACTGTCGAAA CTTTGACTAT TTCTCCACCA CCACCACTGT
 48  L  Q  R   E  I  E  I   M  R  G   L  R  H   P  N  I  V   H  M  L   D  S  F   E  T  D  K   E  V  V   V  V  T

401 GACTATGCTG AGGGAGAGCT CTTTCAGATC CTAGAAGATG ACGGAAAACT TCCTGAAGAC CAGGTTCAGG CCATTGCTGC CCAGTTGGTG TCAGCCCTGT
    CTGATACGAC TCCCTCTCGA GAAAGTCTAG GATCTTCTAC TGCCTTTTGA AGGACTTCTG GTCCAAGTCC GGTAACGACG GGTCAACCAC AGTCGGGACA
 81 D  Y  A  E   G  E  L   F  Q  I   L  E  D  D   G  K  L   P  E  D   Q  V  Q  A   I  A  A   Q  L  V   S  A  L  Y

501 ACTATCTGCA TTCCCACCGC ATCCTACACC GAGATATGAA GCCTCAGAAC ATCCTCCTCG CCAAGGGTGG TGGCATCAAG CTCTGTGACT TTGGATTTGC
    TGATAGACGT AAGGGTGGCG TAGGATGTGG CTCTATACTT CGGAGTCTTG TAGGAGGAGC GGTTCCCACC ACCGTAGTTC GAGACACTGA AACCTAAACG
115   Y  L  H   S  H  R   I  L  H  R   D  M  K   P  Q  N   I  L  L  A   K  G  G   G  I  K   L  C  D  F   G  F  A

601 CCGGGCTATG AGCACCAATA CAATGGTGCT GACATCCATC AAAGGCACAC CACTCTATAT GTCTCCAGAG CTGGTGGAGG AGCGACCATA CGACCACACA
    GGCCCGATAC TCGTGGTTAT GTTACCACGA CTGTAGGTAG TTTCCGTGTG GTGAGATATA CAGAGGTCTC GACCACCTCC TCGCTGGTAT GCTGGTGTGT
148   R  A  M   S  T  N  T   M  V  L   T  S  I   K  G  T  P   L  Y  M   S  P  E   L  V  E  E   R  P  Y   D  H  T

701 GCGGACCTCT GGTCTGTTGG CTGCATACTA TATGAACTGG CAGTAGGCAC CCCTCCCTTC TATGCTACAA GCATCTTTCA GCTGGTCAGC CTCATTCTCA
    CGCCTGGAGA CCAGACAACC GACGTATGAT ATACTTGACC GTCATCCGTG GGGAGGGAAG ATACGATGTT CGTAGAAAGT CGACCAGTCG GAGTAAGAGT
181 A  D  L  W   S  V  G   C  I  L   Y  E  L  A   V  G  T   P  P  F   Y  A  T  S   I  F  Q   L  V  S   L  I  L  K
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FIG. 1A

28. A method of screening for antagonist or agonist molecule of *fused* biological activity comprising:

(a) exposing a *fused* substrate and a compound having *fused* biological activity to a candidate antagonist or agonist; and

(b) analyzing the substrate to assess the level and/or identity of phosphorylation; and

comparing the results to control reactions which were not exposed to the candidate molecule.

29. A method of diagnosing to determine whether a particular disorder is modulated by hedgehog signaling, comprising:

(a) culturing test cells or tissues;

(b) administering a compound which can inhibit *fused* modulated *hedgehog* signaling; and

(c) measuring the degree of kinase attenuation on the *fused* substrate in cell lysates or hedgehog mediated phenotypic effects in the test cells.

15. The host cell of claim 14 which is *Saccharomyces cerevisiae*.

16. A process for producing vertebrate *fused* polypeptides comprising culturing the host cell of claim 9 under conditions suitable for expression of vertebrate *fused* and recovering vertebrate *fused* from the cell culture.

17. Isolated native sequence human *fused* polypeptide comprising amino acid residues 1 to 1315 of Fig. 1.

18. Isolated native sequence human *fused* polypeptide encoded by the nucleotide deposited under accession number ATCC 209637 having *fused* biological activity.

19. A chimeric molecule comprising vertebrate *fused* polypeptide fused to a heterologous amino acid sequence.

20. The chimeric molecule of claim 19 wherein said heterologous amino acid sequence is an epitope tag sequence.

21. The chimeric molecule of claim 22 wherein said heterologous amino acid sequence is a constant region of an immunoglobulin.

22. An antagonist of vertebrate fused which biocks, prevents, inhibits and/or neutralizes the normal functioning of *fused* in the *Hh* signaling pathway.

23. The antagonist of claim 22 which is a small bioorganic moiecule.

24. The antagonist of claim 22 which is an antisense nucleotide.

25. An agonist of vertebrate *fused* with stimulates or enhances the normal functioning of *fused* in the *Hh* signaling pathway.

26. The agonist of claim 25 which is a small bioorganic molecule.

27. A method of screening for antagonists or agonists of *fused* biological activity comprising:

(a) exposing the fused expressing target cells in culture to a candidate compound; and

(b) analyzing cell lysates to assess the level and/or identity of phosphorylation; or

(c) scoring phenotypic or functional changes in treated cells;

and comparing the results to control cells which were not exposed to the candidate compound.

What is claimed is:

1. Isolated nucleic acid comprising DNA having at least a 95% sequence identity to (a) a DNA molecule encoding a vertebrate *fused* polypeptide comprising the sequence of amino acids 1 to about 260 of Fig. 1 (SEQ ID NO:24), or (b) the complement of (a); and encoding a polypeptide having *fused* biological activity.

2. The isolated nucleic acid of claim 1 comprising DNA having at least a 95% sequence identity to (a) a DNA molecule encoding a human *fused* polypeptide comprising the sequence of amino acids 1 to 1315 (SEQ ID NO: 2) of Fig. 1, or (b) the complement of the DNA molecule of (a).

3. The isolated nucleic acid of claim 1 comprising DNA encoding a vertebrate *fused* polypeptide having amino acid residues 1 to 260 of Fig. 1 (SEQ ID NO:24).

4. The isolated nucleic acid of claim 1 comprising DNA encoding a vertebrate *fused* polypeptide having a lysine at amino acid position 33.

5. An isolated nucleic acid comprising DNA having at least a 95% sequence identity to (a) a DNA molecule encoding the same mature polypeptide encoded by the cDNA in ATCC Deposit No. 209637, or (b) the complement of the DNA molecule of (a).

6. The isolated nucleic acid of claim 5 comprising human *fused* encoding sequence of the cDNA in ATCC deposit No. 209637, or a sequence which hybridizes thereto under stringent conditions.

7. A vector comprising the nucleic acid of claim 1.

8. The vector of claim 7 operably linked to control sequences recognized by a host cell transformed with the vector.

9. A host cell transformed with the vector of claim 8.

10. The host cell of claim 9 which is mammalian.

11. The host cell of claim 10 wherein said cell is a CHO cell.

12. The host cell of claim 9 which is prokaryotic.

13. The host cell of claim 12 wherein said cell is an *E. coli*.

14. The host cell of claim 9 wherein said cell is a yeast cell.

produced in the ascites can be accomplished using ammonium sulfate precipitation, followed by gel exclusion chromatography. Alternatively, affinity chromatography based upon binding of antibody to protein A or protein G can be employed.

Deposit of Material

The following materials have been deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD, USA (ATCC):

Designation:	ATCC Dep. No.	Deposit Date
pRK5tkneo.hFused-1272	209637	2/19/98

This deposit was made under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purpose of Patent Procedure and the Regulations thereunder (Budapest Treaty). This assures maintenance of a viable culture of the deposit for 30 years from the date of deposit. The deposit will be made available by ATCC under the terms of the Budapest Treaty, and subject to an agreement between Genentech, Inc. and ATCC, which assures permanent and unrestricted availability of the progeny of the culture of the deposit to the public upon issuance of the pertinent U.S. patent or upon laying open to the public of any U.S. or foreign patent application, whichever comes first, and assures availability of the progeny to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC §122 and the Commissioner's rules pursuant thereto (including 37 CFR § 1.14 with particular reference to 886 OG 638).

The assignee of the present application has agreed that if a culture of the materials on deposit should die or be lost or destroyed when cultivated under suitable conditions, the materials will be promptly replaced on notification with another of the same. Availability of the deposited material is not to be construed as a license to practice the invention in contravention of the rights granted under the authority of any government in accordance with its patent laws.

The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the invention. The present invention is not to be limited in scope by the construct deposited, since the deposited embodiment is intended as a single illustration of certain aspects of the invention and any constructs that are functionally equivalent are within the scope of this invention. The deposit of material herein does not constitute an admission that the written description herein contained is inadequate to enable the practice of any aspect of the invention, including the best mode thereof, nor is it to be construed as limiting the scope of the claims to the specific illustrations that it represents. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and fall within the scope of the appended claims.

baseline A_{280} with loading buffer, at which point fraction collection is started. Next, the column is washed with a secondary wash buffer (50 mM phosphate; 300 mM NaCl, 10% Glycerol, pH 6.0), which elutes nonspecifically bound protein. After reaching A_{280} baseline again, the column is developed with a 0 to 500 mM Imidazole gradient in the secondary wash buffer. One mL fractions are collected and analyzed by SDS-PAGE and silver staining or western blot with Ni^{2+}-NTA-conjugated to alkaline phosphatase (Qiagen). Fractions containing the eluted His_{10}-tagged vertebrate *fused* are pooled and dialyzed against loading buffer.

Alternatively, purification of the IgG tagged (or Fc tagged) vertebrate *fused* can be performed using known chromatography techniques, including for instance, Protein A or protein G column chromatography

EXAMPLE 15

Preparation of Antibodies that Bind Vertebrate *fused*

This example illustrates preparation of monoclonal antibodies, which can specifically bind vertebrate *fused*.

Techniques for producing the monoclonal antibodies are known in the art and are described, for instance, in Goding, supra. Immunogens that may be employed include purified vertebrate *fused*, fusion proteins containing vertebrate *fused*, and cells expressing recombinant vertebrate *fused* on the cell surface. Selection of the immunogen can be made by the skilled artisan without undue experimentation.

Mice, such as Balb/c, are immunized with the vertebrate *fused* immunogen emulsified in complete Freund's adjuvant and injected subcutaneously or intraperitoneally in an amount from 1-100 micrograms. Alternatively, the immunogen is emulsified in MPL-TDM adjuvant (Ribi Immunochemical Research, Hamilton, MT) and injected into the animal's hind foot pads. The immunized mice are then boosted 10 to 12 days later with additional immunogen emulsified in the selected adjuvant. Thereafter, for several weeks, the mice may also be boosted with additional immunization injections. Serum samples may be periodically obtained from the mice by retro-orbital bleeding for testing in ELISA assays to detect vertebrate *fused* antibodies.

After a suitable antibody titer has been detected, the animals "positive" for antibodies can be injected with a final intravenous injection of vertebrate *fused*. Three to four days later, the mice are sacrificed and the spleen cells are harvested. The spleen cells are then fused (using 35% polyethylene glycol) to a selected murine myeloma cell line such as P3X63AgU.1, available from ATCC, No. CRL 1597. The fusions generate hybridoma cells which can then be plated in 96 well tissue culture plates containing HAT (hypoxanthine, aminopterin, and thymidine) medium to inhibit proliferation of non-fused cells, myeloma hybrids, and spleen cell hybrids.

The hybridoma cells will be screened in an ELISA for reactivity against vertebrate *fused*. Determination of "positive" hybridoma cells secreting the desired monoclonal antibodies against vertebrate *fused* is within the skill in the art.

The positive hybridoma cells can be injected intraperitoneally into syngeneic Balb/c mice to produce ascites containing the anti- vertebrate *fused* monoclonal antibodies. Alternatively, the hybridoma cells can be grown in tissue culture flasks or roller bottles. Purification of the monoclonal antibodies

plasmid, together with DNA encoding the ADH2/GAPDH promoter, the yeast alpha-factor secretory signal/leader sequence, and linker sequences (if needed) for expression of vertebrate *fused*.

Yeast cells, such as yeast strain AB110, can then be transformed with the expression plasmids described above and cultured in selected fermentation media. The transformed yeast supernatants can be analyzed by precipitation with 10% trichloroacetic acid and separation by SDS-PAGE, followed by staining of the gels with Coomassie Blue stain.

Recombinant vertebrate *fused* can subsequently be isolated and purified by removing the yeast cells from the fermentation medium by centrifugation and then concentrating the medium using selected cartridge filters. The concentrate containing vertebrate *fused* may further be purified using selected column chromatography resins.

EXAMPLE 14

Expression of vertebrate *fused* in Baculovirus-Infected Insect Cells

The following method describes recombinant expression of vertebrate *fused* in Baculovirus-infected insect cells.

The vertebrate *fused* is fused upstream of an epitope tag contained within a baculovirus expression vector. Such epitope tags include poly-his tags and immunoglobulin tags (like Fc regions of IgG). A variety of plasmids may be employed, including plasmids derived from commercially available plasmids such as pVL1393 (Novagen). Briefly, the vertebrate *fused* or the desired portion of the vertebrate *fused* (such as the sequence encoding the extracellular domain of a transmembrane protein) is amplified by PCR with primers complementary to the 5' and 3' regions. The 5' primer may incorporate flanking (selected) restriction enzyme sites. The product is then digested with those selected restriction enzymes and subcloned into the expression vector.

Recombinant baculovirus is generated by co-transfecting the above plasmid and BaculoGold™ virus DNA (Pharmingen) into *Spodoptera frugiperda* ("Sf9") cells (ATCC CRL 1711) using lipofectin (commercially available from GIBCO-BRL). After 4 - 5 days of incubation at 28°C, the released viruses are harvested and used for further amplifications. Viral infection and protein expression is performed as described by O'Reilley *et al., Baculovirus expression vectors; A Laboratory Manual*, Oxford: Oxford University Press (1994).

Expressed poly-his tagged vertebrate *fused* can then be purified, for example, by Ni^{2+}-chelate affinity chromatography as follows. Extracts are prepared from recombinant virus-infected Sf9 cells as described by Rupert et al., Nature, 362:175-179 (1993). Briefly, Sf9 cells are washed, resuspended in sonication buffer (25 mL Hepes, pH 7.9; 12.5 mM $MgCl_2$; 0.1 mM EDTA; 10% Glycerol; 0.1% NP-40; 0.4 M KCl), and sonicated twice for 20 seconds on ice. The sonicates are cleared by centrifugation, and the supernatant is diluted 50-fold in loading buffer (50 mM phosphate, 300 mM NaCl, 10% Glycerol, pH 7.8) and filtered through a 0.45 μm filter. A Ni^{2+}-NTA agarose column (commercially available from Qiagen) is prepared with a bed volume of 5 mL, washed with 25 mL of water and equilibrated with 25 mL of loading buffer. The filtered cell extract is loaded onto the column at 0.5 mL per minute. The column is washed to

Approximately 24 hours after the transfections, the culture medium is removed and replaced with culture medium (alone) or culture medium containing 200 µCi/ml ^{35}S-cysteine and 200 µCi/ml ^{35}S-methionine. After a 12 hour incubation, the conditioned medium is collected, concentrated on a spin filter, and loaded onto a 15% SDS gel. The processed gel may be dried and exposed to film for a selected period of time to reveal the presence of vertebrate *fused* polypeptide. The cultures containing transfected cells may undergo further incubation (in serum free medium) and the medium is tested in selected bioassays.

In an alternative technique, vertebrate *fused* may be introduced into 293 cells transiently using the dextran sulfate method described by Somparyrac *et al., Proc. Natl. Acad. Sci.*, 12:7575 (1981). 293 cells are grown to maximal density in a spinner flask and 700 µg pRK5-*fused* DNA is added. The cells are first concentrated from the spinner flask by centrifugation and washed with PBS. The DNA-dextran precipitate is incubated on the cell pellet for four hours. The cells are treated with 20% glycerol for 90 seconds, washed with tissue culture medium, and re-introduced into the spinner flask containing tissue culture medium, 5 µg/ml bovine insulin and 0.1 µg/ml bovine transferrin. After about four days, the conditioned media is centrifuged and filtered to remove cells and debris. The sample containing expressed vertebrate *fused* can then be concentrated and purified by any selected method, such as dialysis and/or column chromatography.

In another embodiment, vertebrate *fused* can be expressed in CHO cells. The pSUi-*fused* can be transfected into CHO cells using known reagents such as $CaPO_4$ or DEAE-dextran. As described above, the cell cultures can be incubated, and the medium replaced with culture medium (alone) or medium containing a radiolabel such as ^{35}S-methionine. After determining the presence of vertebrate *fused* polypeptide, the culture medium may be replaced with serum free medium. Preferably, the cultures are incubated for about 6 days, and then the conditioned medium is harvested. The medium containing the expressed vertebrate *fused* can then be concentrated and purified by any selected method.

Epitope-tagged vertebrate *fused* may also be expressed in host CHO cells. The vertebrate *fused* may be subcloned out of the pRK5 vector. The subclone insert can undergo PCR to fuse in frame with a selected epitope tag such as a poly-his tag into an expression vector. The poly-his tagged vertebrate *fused* insert can then be subcloned into a SV40 driven vector containing a selection marker such as DHFR for selection of stable clones. Finally, the CHO cells can be transfected (as described above) with the SV40 driven vector. Labeling may be performed, as described above, to verify expression. The culture medium containing the expressed poly-His tagged vertebrate *fused* can then be concentrated and purified by any selected method, such as by Ni^{2+}-chelate affinity chromatography.

EXAMPLE 13

Expression of vertebrate *fused* in Yeast

The following method describes recombinant expression of vertebrate *fused* in yeast.

First, yeast expression vectors are constructed for intracellular production or secretion of vertebrate *fused* from the ADH2/GAPDH promoter. DNA encoding vertebrate *fused*, a selected signal peptide and the promoter is inserted into suitable restriction enzyme sites in the selected plasmid to direct intracellular expression of vertebrate *fused*. For secretion, DNA encoding vertebrate *fused* can be cloned into the selected

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990 995

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Cys Thr Phe Asn Leu Glu Arg Ser Gln Thr Ser Asp Ser Leu Gln
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Ile Lys Leu Cys Asp Phe Gly Phe Ala Arg Ala Met Ser
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acc aat aca atg gtg ctg aca tcc atc aaa ggc aca cca 607
Thr Asn Thr Met Val Leu Thr Ser Ile Lys Gly Thr Pro
        155                 160

ctc tat atg tct cca gag ctg gtg gag gag cga cca tac 646
Leu Tyr Met Ser Pro Glu Leu Val Glu Glu Arg Pro Tyr
165                 170                 175

gac cac aca gcg gac ctc tgg tct gtt ggc tgc ata cta 685
Asp His Thr Ala Asp Leu Trp Ser Val Gly Cys Ile Leu
        180                 185                 190

tat gaa ctg gca gta ggc acc cct ccc ttc tat gct aca 724
Tyr Glu Leu Ala Val Gly Thr Pro Pro Phe Tyr Ala Thr
            195                 200

agc atc ttt cag ctg gtc agc ctc att ctc aag gac cct 763
Ser Ile Phe Gln Leu Val Ser Leu Ile Leu Lys Asp Pro
    205                 210                 215

gtg cgc tgg ccc tca acc atc agt ccc tgc ttt aag aac 802
Val Arg Trp Pro Ser Thr Ile Ser Pro Cys Phe Lys Asn
        220                 225

ttc ctg cag gga ctg ctc acc aaa gac cca cgg cag cga 841
Phe Leu Gln Gly Leu Leu Thr Lys Asp Pro Arg Gln Arg
230                 235                 240

ctg tcc tgg cca gac ctc tta tat cac ccc ttt att gct 880
Leu Ser Trp Pro Asp Leu Leu Tyr His Pro Phe Ile Ala
        245                 250                 255

ggt cat gtc acc ata ata act gag cca gca ggc cca gat 919
Gly His Val Thr Ile Ile Thr Glu Pro Ala Gly Pro Asp
            260                 265

ttg ggg acc cca ttc acc agc cgc cta ccc cca gaa ctt 958
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Leu Gly Thr Pro Phe Thr Ser Arg Leu Pro Pro Glu Leu
    270                 275                 280

cag gtc cta aag gac gaa cag gcc cat cgg ttg gcc ccc 997
Gln Val Leu Lys Asp Glu Gln Ala His Arg Leu Ala Pro
            285                 290

aag ggt aat cag tct cgc atc ttg act cag gcc tat aaa 1036
Lys Gly Asn Gln Ser Arg Ile Leu Thr Gln Ala Tyr Lys
295                 300                 305

cgc atg gct gag gag gcc atg cag aag aaa cat cag aac 1075
Arg Met Ala Glu Glu Ala Met Gln Lys Lys His Gln Asn
        310                 315                 320

aca gga cct gcc ctt gag caa gag gac aag acc agc aag 1114
Thr Gly Pro Ala Leu Glu Gln Glu Asp Lys Thr Ser Lys
                325                 330

gtg gct cct ggc aca gcc cct ctg ccc aga ctc ggg gcc 1153
Val Ala Pro Gly Thr Ala Pro Leu Pro Arg Leu Gly Ala
    335                 340                 345

act cct cag gaa tca agc ctc ctg gcc ggg atc tta gcc 1192
Thr Pro Gln Glu Ser Ser Leu Leu Ala Gly Ile Leu Ala
            350                 355

tca gaa ttg aag agc agc tgg gct aaa tca ggg act gga 1231
Ser Glu Leu Lys Ser Ser Trp Ala Lys Ser Gly Thr Gly
360                 365                 370

gag gtg ccc tct gca cct cgg gaa aac cgg acc acc cca 1270
Glu Val Pro Ser Ala Pro Arg Glu Asn Arg Thr Thr Pro
        375                 380                 385

gat tgt gaa cga gca ttc cca gag gag agg cca gag gtg 1309
Asp Cys Glu Arg Ala Phe Pro Glu Glu Arg Pro Glu Val
                390                 395

ctg ggc cag cgg agc act gat gta gtg gac ctg gaa aat 1348
Leu Gly Gln Arg Ser Thr Asp Val Val Asp Leu Glu Asn
    400                 405                 410

gag gag cca gac agt gac aat gag tgg cag cac ctg cta 1387
Glu Glu Pro Asp Ser Asp Asn Glu Trp Gln His Leu Leu
            415                 420

gag acc act gag cct gtg cct att caa ctg aag gct cct 1426
Glu Thr Thr Glu Pro Val Pro Ile Gln Leu Lys Ala Pro
425                 430                 435

ctc acc ttg ctg tgt aat cct gac ttc tgc cag cgc atc 1465
Leu Thr Leu Leu Cys Asn Pro Asp Phe Cys Gln Arg Ile
        440                 445                 450

cag agt cag ctg cat gaa gct gga ggg cag atc ctg aaa 1504
Gln Ser Gln Leu His Glu Ala Gly Gly Gln Ile Leu Lys
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Gly Ile Leu Glu Gly Ala Ser His Ile Leu Pro Ala Phe
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Arg Val Leu Ser Ser Leu Leu Ser Ser Cys Ser Asp Ser
480 485

gtt gcc ttg tat tcc ttc tgc cgg gag gca ggg ctt cct 1621
Val Ala Leu Tyr Ser Phe Cys Arg Glu Ala Gly Leu Pro
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Gly Leu Leu Leu Ser Leu Leu Arg His Ser Gln Glu Ser
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Asn Ser Leu Gln Gln Gln Ser Trp Tyr Gly Thr Phe Leu
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cag gac ctg atg gct gtg att cag gcc tac ttt gcc tgt 1738
Gln Asp Leu Met Ala Val Ile Gln Ala Tyr Phe Ala Cys
530 535 540

acc ttc aat ctg gag agg agc cag aca agt gac agc ctg 1777
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Gln Val Phe Gln Glu Ala Ala Asn Leu Phe Leu Asp Leu
555 560 565

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Leu Gly Lys Leu Leu Ala Gln Pro Asp Asp Ser Glu Gln
570 575 580

act ttg cag agg gac agc ctt atg tgc ttt act gtc ctg 1894
Thr Leu Gln Arg Asp Ser Leu Met Cys Phe Thr Val Leu
585 590

tgc gaa gcc atg gat ggg aac agc cgg gcc atc tcc aaa 1933
Cys Glu Ala Met Asp Gly Asn Ser Arg Ala Ile Ser Lys
595 600 605

gcc ttt tac tcc agc ttg ctg acg aca cag cag gtt gtc 1972
Ala Phe Tyr Ser Ser Leu Leu Thr Thr Gln Gln Val Val
610 615

ttg gat ggg ctc ctt cat ggc ttg aca gtt cca cag ctc 2011
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620 625 630

cct gtc cac act ccc caa ggt aac cag agt gga gaa ggg 2050
Pro Val His Thr Pro Gln Gly Asn Gln Ser Gly Glu Gly
635 640 645

agg ttc tct t gacttacttg ttgcataggt caggctccgc 2090
Arg Phe Ser
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tctttctatt gccatcacct agatcgcacc tggcatttag taggtgctca 2140

ataaataact gtgaactgag agaatgaatg gggatctgag ggaaacaaac 2190

agacctcatc ctgcattctt cccactccct taggttccct actcctgctg 2240

ccatgtcggt gagtactggt gctattgtct agggcaagag cctcaggcct 2290

ttgg agt tac tct ttg ctt ttc tcc aca gga gcc ccg 2327
Ser Tyr Ser Leu Leu Phe Ser Thr Gly Ala Pro
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caa gtg agc cag cca ctg cga gag cag agt gag gat ata 2366
Gln Val Ser Gln Pro Leu Arg Glu Gln Ser Glu Asp Ile
15 20

cct gga gcc att tcc tct gcc ctg gca gcc ata tgc act 2405
Pro Gly Ala Ile Ser Ser Ala Leu Ala Ala Ile Cys Thr
25 30 35

gct cct gtg gga ctg ccc gac tgc tgg gat gcc aag gag 2444
Ala Pro Val Gly Leu Pro Asp Cys Trp Asp Ala Lys Glu
40 45 50

cag gtc tgt tgg cat ttg gca aat cag cta act gaa gac 2483
Gln Val Cys Trp His Leu Ala Asn Gln Leu Thr Glu Asp
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Ser Ser Gln Leu Arg Pro Ser Leu Ile Ser Gly Leu Gln
65 70 75

cat ccc atc ctg tgc ctg cac ctt ctc aag gtt cta tac 2561
His Pro Ile Leu Cys Leu His Leu Leu Lys Val Leu Tyr
80 85

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Ser Cys Cys Leu Val Ser Glu Gly Leu Cys Arg Leu Leu
90 95 100

ggg cag gag ccc ctg gcc ttg gaa tcc ctg ttt atg ttg 2639
Gly Gln Glu Pro Leu Ala Leu Glu Ser Leu Phe Met Leu
105 110 115

att cag ggc aag gta aaa gta gta gat tgg gaa gag tct 2678
Ile Gln Gly Lys Val Lys Val Val Asp Trp Glu Glu Ser
120 125

act gaa gtg aca ctc tac ttc ctc tcc ctt ctt gtc ttt 2717
Thr Glu Val Thr Leu Tyr Phe Leu Ser Leu Leu Val Phe
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Arg Leu Gln Asn Leu Pro Cys Gly Met Glu Lys Leu Gly
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Ser Asp Val Ala Thr Leu Phe Thr His Ser His Val Val
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tct ctt gtg agt gca gca gcc tgt cta ttg gga cag ctt 2834
Ser Leu Val Ser Ala Ala Ala Cys Leu Leu Gly Gln Leu
        170                     175                 180

ggt cag caa ggg gtg acc ttt gac ctc cag ccc atg gaa 2873
Gly Gln Gln Gly Val Thr Phe Asp Leu Gln Pro Met Glu
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tgg atg gct gca gcc aca cat gcc ttg tct gcc cct gca 2912
Trp Met Ala Ala Ala Thr His Ala Leu Ser Ala Pro Ala
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Glu Val Arg Leu Thr Pro Pro Gly Ser Cys Gly Phe Tyr
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220                 225                 230

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Gln Gly Lys Ala Ser Leu Ile Arg Asp Met Ser Ser Ser
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Glu Met Trp Thr Val Leu Trp His Arg Phe Ser Met Val
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Leu Arg Leu Pro Glu Glu Ala Ser Ala Gln Glu Gly Glu
    260                     265                     270

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Thr Leu Ile Ser Pro Gln Gly Met Ala Ala Leu Leu Ser
285                 290                 295

ctg gcc atg gcc acc ttt acc cag gag ccc cag tta tgc 3224
Leu Ala Met Ala Thr Phe Thr Gln Glu Pro Gln Leu Cys
        300                     305                 310

ctg agc tgc ctg tcc cag cat gga agt atc ctc atg tcc 3263
Leu Ser Cys Leu Ser Gln His Gly Ser Ile Leu Met Ser
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atc ctg aag cat ctg ctt tgc ccc agc ttc ctg aat caa 3302
Ile Leu Lys His Leu Leu Cys Pro Ser Phe Leu Asn Gln
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325                 330                 335

ctg cgc cag gcg cct cat ggg tct gag ttt ctc cct gtc 3341
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Val Val Leu Ser Val Cys Gln Leu Leu Cys Phe Pro Phe
350                 355                 360

gcg ctg gac atg gat gct gac ctc ctt ata gtt gtc ttg 3419
Ala Leu Asp Met Asp Ala Asp Leu Leu Ile Val Val Leu
        365                 370                 375

gcc gac ctc agg gac tca gaa gtt gca gcc cat ctg ctg 3458
Ala Asp Leu Arg Asp Ser Glu Val Ala Ala His Leu Leu
                380                 385

cag gtc tgc tgc tac cat ctt ccg ttg atg caa gtg gag 3497
Gln Val Cys Cys Tyr His Leu Pro Leu Met Gln Val Glu
    390                 395                 400

ctg ccc atc agc ctt ctc aca cgc ctg gcc ctc atg gat 3536
Leu Pro Ile Ser Leu Leu Thr Arg Leu Ala Leu Met Asp
            405                 410

ccc acc tct ctc aac cag ttt gtg aac aca gtg tct gcc 3575
Pro Thr Ser Leu Asn Gln Phe Val Asn Thr Val Ser Ala
415                 420                 425

tcc cct aga acc atc gtc tcg ttt ctc tca gtt gcc ctc 3614
Ser Pro Arg Thr Ile Val Ser Phe Leu Ser Val Ala Leu
        430                 435                 440

ctg agt gac cag cca ctg ttg acc tcc gac ctt ctc tct 3653
Leu Ser Asp Gln Pro Leu Leu Thr Ser Asp Leu Leu Ser
                445                 450

ctg ctg gcc cat act gcc agg gtc ctg tct ccc agc cac 3692
Leu Leu Ala His Thr Ala Arg Val Leu Ser Pro Ser His
    455                 460                 465

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Leu Ser Phe Ile Gln Glu Leu Leu Ala Gly Ser Asp Glu
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Ser Tyr Arg Pro Leu Arg Ser Leu Leu Gly His Pro Glu
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Asn Ser Val Arg Ala His Thr Tyr Arg Leu Leu Gly His
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Pro Ala Leu Ala Ala Ala Val Pro Ser Met Thr Gln Leu
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Leu Gly Asp Pro Gln Ala Gly Ile Arg Arg Asn Val Ala
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Ala Leu Ile Ala Leu Arg Ser Leu Gln Gln Glu Pro Gly
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Ile His Gln Val Leu Val Ser Leu Gly Ala Ser Glu Lys
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Leu Ser Leu Leu Ser Leu Gly Asn Gln Ser Leu Pro His
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Ser Ser Pro Arg Pro Ala Ser Ala Lys His Cys Arg Lys
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ctc att cac ctc ctg agg cca gcc cat agc atg t 4350
Leu Ile His Leu Leu Arg Pro Ala His Ser Met
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Arg Asn Leu Gln Arg Glu Ile Glu Ile Met Arg Gly Leu Arg His
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Pro Asn Ile Val His Met Leu Asp Ser Phe Glu Thr Asp Lys Glu
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Val Val Val Val Thr Asp Tyr Ala Glu Gly Glu Leu Phe Gln Ile
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Leu Glu Asp Asp Gly Lys Leu Pro Glu Asp Gln Val Gln Ala Ile
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Ala Ala Gln Leu Val Ser Ala Leu Tyr Tyr Leu His Ser His Arg
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Ile Leu His Arg Asp Met Lys Pro Gln Asn Ile Leu Leu Ala Lys

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Gly Gly Gly Ile Lys Leu Cys Asp Phe Gly Phe Ala Arg Ala Met
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Ser Thr Asn Thr Met Val Leu Thr Ser Ile Lys Gly Thr Pro Leu
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Tyr Met Ser Pro Glu Leu Val Glu Glu Arg Pro Tyr Asp His Thr
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Ala Asp Leu Trp Ser Val Gly Cys Ile Leu Tyr Glu Leu Ala Val
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Gly Thr Pro Pro Phe Tyr Ala Thr Ser Ile Phe Gln Leu Val Ser
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Leu Ile Leu Lys Asp Pro Val Arg Trp Pro Ser Thr Ile Ser Pro
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Cys Phe Lys Asn Phe Leu Gln Gly Leu Leu Thr Lys Asp Pro Arg
230 235 240

Gln Arg Leu Ser Trp Pro Asp Leu Leu Tyr His Pro Phe Ile Ala
245 250 255

Gly His Val Thr Ile Ile Thr Glu Pro Ala Gly Pro Asp Leu Gly
260 265 270

Thr Pro Phe Thr Ser Arg Leu Pro Pro Glu Leu Gln Val Leu Lys
275 280 285

Asp Glu Gln Ala His Arg Leu Ala Pro Lys Gly Asn Gln Ser Arg
290 295 300

Ile Leu Thr Gln Ala Tyr Lys Arg Met Ala Glu Glu Ala Met Gln
305 310 315

Lys Lys His Gln Asn Thr Gly Pro Ala Leu Glu Gln Glu Asp Lys
320 325 330

Thr Ser Lys Val Ala Pro Gly Thr Ala Pro Leu Pro Arg Leu Gly
335 340 345

Ala Thr Pro Gln Glu Ser Ser Leu Leu Ala Gly Ile Leu Ala Ser
350 355 360

Glu Leu Lys Ser Ser Trp Ala Lys Ser Gly Thr Gly Glu Val Pro
365 370 375

Ser Ala Pro Arg Glu Asn Arg Thr Thr Pro Asp Cys Glu Arg Ala
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Phe Pro Glu Glu Arg Pro Glu Val Leu Gly Gln Arg Ser Thr Asp
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Val Val Asp Leu Glu Asn Glu Glu Pro Asp Ser Asp Asn Glu Trp

410 415 420

Gln His Leu Leu Glu Thr Thr Glu Pro Val Pro Ile Gln Leu Lys
425 430 435

Ala Pro Leu Thr Leu Leu Cys Asn Pro Asp Phe Cys Gln Arg Ile
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Gln Ser Gln Leu His Glu Ala Gly Gly Gln Ile Leu Lys Gly Ile
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Leu Glu Gly Ala Ser His Ile Leu Pro Ala Phe Arg Val Leu Ser
470 475 480

Ser Leu Leu Ser Ser Cys Ser Asp Ser Val Ala Leu Tyr Ser Phe
485 490 495

Cys Arg Glu Ala Gly Leu Pro Gly Leu Leu Leu Ser Leu Leu Arg
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His Ser Gln Glu Ser Asn Ser Leu Gln Gln Gln Ser Trp Tyr Gly
515 520 525

Thr Phe Leu Gln Asp Leu Met Ala Val Ile Gln Ala Tyr Phe Ala
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Cys Thr Phe Asn Leu Glu Arg Ser Gln Thr Ser Asp Ser Leu Gln
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Val Phe Gln Glu Ala Ala Asn Leu Phe Leu Asp Leu Leu Gly Lys
560 565 570

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590 595 600

Ser Arg Ala Ile Ser Lys Ala Phe Tyr Ser Ser Leu Leu Thr Thr
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Gln Gln Val Val Leu Asp Gly Leu Leu His Gly Leu Thr Val Pro
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Arg Phe Ser
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Ile Gly Glu Gly Ser Phe Gly Arg Val Tyr Lys Gly Arg
 10                  15                  20

aga aaa tac agt gct cag gtc gtg gcc ctg aag ttc atc 219
Arg Lys Tyr Ser Ala Gln Val Val Ala Leu Lys Phe Ile
        25                  30                  35

cca aaa ttg ggg cgc tca gag aag gag ctg agg aat ttg 258
Pro Lys Leu Gly Arg Ser Glu Lys Glu Leu Arg Asn Leu
                40                  45

caa cga gag att gaa ata atg cgg ggt ctg cgg cat ccc 297
Gln Arg Glu Ile Glu Ile Met Arg Gly Leu Arg His Pro
    50                  55                  60

aac att gtg cat atg ctt gac agc ttt gaa act gat aaa 336
Asn Ile Val His Met Leu Asp Ser Phe Glu Thr Asp Lys
            65                  70

gag gtg gtg gtg gtg aca gac tat gct gag gga gag ctc 375
Glu Val Val Val Val Thr Asp Tyr Ala Glu Gly Glu Leu
 75                  80                  85

ttt cag atc cta gaa gat gac gga aaa ctt cct gaa gac 414
Phe Gln Ile Leu Glu Asp Asp Gly Lys Leu Pro Glu Asp
        90                  95                  100

cag gtt cag gcc att gct gcc cag ttg gtg tca gcc ctg 453
Gln Val Gln Ala Ile Ala Ala Gln Leu Val Ser Ala Leu
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tac tat ctg cat tcc cac cgc atc cta cac cga gat atg 492
Tyr Tyr Leu His Ser His Arg Ile Leu His Arg Asp Met
    115                 120                 125

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Lys Pro Gln Asn Ile Leu Leu Ala Lys Gly Gly Gly Ile
            130                 135

aag ctc tgt gac ttt gga ttt gcc cgg gct atg agc acc 570
Lys Leu Cys Asp Phe Gly Phe Ala Arg Ala Met Ser Thr
140                 145                 150

aat aca atg gtg ctg aca tcc atc aaa ggc aca cca ctc 609
Asn Thr Met Val Leu Thr Ser Ile Lys Gly Thr Pro Leu
        155                 160                 165

tat atg tct cca gag ctg gtg gag gag cga cca tac gac 648
Tyr Met Ser Pro Glu Leu Val Glu Glu Arg Pro Tyr Asp
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His Thr Ala Asp Leu Trp Ser Val Gly Cys Ile Leu Tyr
    180                 185                 190

gaa ctg gca gta ggc acc cct ccc ttc tat gct aca agc 726
Glu Leu Ala Val Gly Thr Pro Pro Phe Tyr Ala Thr Ser
            195                 200

atc ttt cag ctg gtc agc ctc att ctc aag gac cct gtg 765
Ile Phe Gln Leu Val Ser Leu Ile Leu Lys Asp Pro Val
205                 210                 215

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Arg Trp Pro Ser Thr Ile Ser Pro Cys Phe Lys Asn Phe
        220                 225                 230

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Leu Gln Gly Leu Leu Thr Lys Asp Pro Arg Gln Arg Leu
                235                 240

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Ser Trp Pro Asp Leu Leu Tyr His Pro Phe Ile Ala Gly
    245                 250                 255

cat gtc acc ata ata act gag cca gca ggc cca gat ttg 921
His Val Thr Ile Ile Thr Glu Pro Ala Gly Pro Asp Leu
            260                 265

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Gly Thr Pro Phe Thr Ser Arg Leu Pro Pro Glu Leu Gln
270                 275                 280

gtc cta aag gac gaa cag gcc cat cgg ttg gcc ccc aag 999
Val Leu Lys Asp Glu Gln Ala His Arg Leu Ala Pro Lys
        285                 290                 295

ggt aat cag tct cgc atc ttg act cag gcc tat aaa cgc 1038
Gly Asn Gln Ser Arg Ile Leu Thr Gln Ala Tyr Lys Arg
                300                 305

atg gct gag gag gcc atg cag aag aaa cat cag aac aca 1077
Met Ala Glu Glu Ala Met Gln Lys Lys His Gln Asn Thr
    310                 315                 320

gga cct gcc ctt gag caa gag gac aag acc agc aag gtg 1116
Gly Pro Ala Leu Glu Gln Glu Asp Lys Thr Ser Lys Val
            325                 330

gct cct ggc aca gcc cct ctg ccc aga ctc ggg gcc act 1155
Ala Pro Gly Thr Ala Pro Leu Pro Arg Leu Gly Ala Thr
335                 340                 345

cct cag gaa tca agc ctc ctg gcc ggg atc tta gcc tca 1194
Pro Gln Glu Ser Ser Leu Leu Ala Gly Ile Leu Ala Ser
        350                 355                 360

gaa ttg aag agc agc tgg gct aaa tca ggg act gga gag 1233
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Glu Leu Lys Ser Ser Trp Ala Lys Ser Gly Thr Gly Glu
                365                      370

gtg ccc tct gca cct cgg gaa aac cgg acc acc cca gat 1272
Val Pro Ser Ala Pro Arg Glu Asn Arg Thr Thr Pro Asp
    375                 380                     385

tgt gaa cga gca ttc cca gag gag agg cca gag gtg ctg 1311
Cys Glu Arg Ala Phe Pro Glu Glu Arg Pro Glu Val Leu
            390                 395

ggc cag cgg agc act gat gta gtg gac ctg gaa aat gag 1350
Gly Gln Arg Ser Thr Asp Val Val Asp Leu Glu Asn Glu
400                 405                 410

gag cca gac agt gac aat gag tgg cag cac ctg cta gag 1389
Glu Pro Asp Ser Asp Asn Glu Trp Gln His Leu Leu Glu
        415                 420                     425

acc act gag cct gtg cct att caa ctg aag gct cct ctc 1428
Thr Thr Glu Pro Val Pro Ile Gln Leu Lys Ala Pro Leu
                430                 435

acc ttg ctg tgt aat cct gac ttc tgc cag cgc atc cag 1467
Thr Leu Leu Cys Asn Pro Asp Phe Cys Gln Arg Ile Gln
    440                 445                     450

agt cag ctg cat gaa gct gga ggg cag atc ctg aaa ggc 1506
Ser Gln Leu His Glu Ala Gly Gly Gln Ile Leu Lys Gly
            455                 460

atc ttg gag ggt gct tcc cac atc ctg cct gca ttc cgg 1545
Ile Leu Glu Gly Ala Ser His Ile Leu Pro Ala Phe Arg
465                 470                 475

gtc ctg agc agt ctt ctc tcc agc tgc agt gat tct gtt 1584
Val Leu Ser Ser Leu Leu Ser Ser Cys Ser Asp Ser Val
        480                 485                     490

gcc ttg tat tcc ttc tgc cgg gag gca ggg ctt cct ggg 1623
Ala Leu Tyr Ser Phe Cys Arg Glu Ala Gly Leu Pro Gly
                495                 500

ctg ctg ctg agt cta ctc agg cac agt cag gag agc aac 1662
Leu Leu Leu Ser Leu Leu Arg His Ser Gln Glu Ser Asn
    505                 510                     515

agc ctc cag cag caa tct tgg tat ggg acc ttc tta cag 1701
Ser Leu Gln Gln Gln Ser Trp Tyr Gly Thr Phe Leu Gln
            520                 525

gac ctg atg gct gtg att cag gcc tac ttt gcc tgt acc 1740
Asp Leu Met Ala Val Ile Gln Ala Tyr Phe Ala Cys Thr
530                 535                 540

ttc aat ctg gag agg agc cag aca agt gac agc ctg cag 1779
Phe Asn Leu Glu Arg Ser Gln Thr Ser Asp Ser Leu Gln
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545 550 555

gtg ttt cag gag gct gcc aac ctt ttt ctg gac ctg ttg 1818
Val Phe Gln Glu Ala Ala Asn Leu Phe Leu Asp Leu Leu
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Gly Lys Leu Leu Ala Gln Pro Asp Asp Ser Glu Gln Thr
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ttg cgg agg gac agc ctt atg tgc ttt act gtc ctg tgc 1896
Leu Arg Arg Asp Ser Leu Met Cys Phe Thr Val Leu Cys
585 590

gaa gcc atg gat ggg aac agc cgg gcc atc tcc aaa gcc 1935
Glu Ala Met Asp Gly Asn Ser Arg Ala Ile Ser Lys Ala
595 600 605

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Phe Tyr Ser Ser Leu Leu Thr Thr Gln Gln Val Val Leu
610 615 620

gat ggg ctc ctt cat ggc ttg aca gtt cca cag ctc cct 2013
Asp Gly Leu Leu His Gly Leu Thr Val Pro Gln Leu Pro
625 630

gtc cac act ccc caa ggt tcc cta ctc ctg ctg cca tgt 2052
Val His Thr Pro Gln Gly Ser Leu Leu Leu Leu Pro Cys
635 640 645

cgg tga g t actggtgcta ttgtctaggg caagagcctc 2090
Arg Xaa
648

aggcctttgg agt tac tct ttg ctt ttc tcc aca gga gcc 2130
Ser Tyr Ser Leu Leu Phe Ser Thr Gly Ala
1 5 10

ccg caa gtg agc cag cca ctg cga gag cag agt gag gat 2169
Pro Gln Val Ser Gln Pro Leu Arg Glu Gln Ser Glu Asp
15 20

ata cct gga gcc att tcc tct gcc ctg gca gcc ata tgc 2208
Ile Pro Gly Ala Ile Ser Ser Ala Leu Ala Ala Ile Cys
25 30 35

act gct cct gtg gga ctg ccc gac tgc tgg gat gcc aag 2247
Thr Ala Pro Val Gly Leu Pro Asp Cys Trp Asp Ala Lys
40 45

gag cag gtc tgt tgg cat ttg gca aat cag cta act gaa 2286
Glu Gln Val Cys Trp His Leu Ala Asn Gln Leu Thr Glu
50 55 60

gac agc agc cag ctc agg cca tcc ctc atc tct ggc ctg 2325
Asp Ser Ser Gln Leu Arg Pro Ser Leu Ile Ser Gly Leu
65 70 75

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cag cat ccc atc ctg tgc ctg cac ctt ctc aag gtt cta 2364
Gln His Pro Ile Leu Cys Leu His Leu Leu Lys Val Leu
                80                      85

tac tcc tgc tgc ctt gtc agt gag ggc ctg tgc cgt ctt 2403
Tyr Ser Cys Cys Leu Val Ser Glu Gly Leu Cys Arg Leu
    90                  95                  100

ctg ggg cag gag ccc ctg gcc ttg gaa tcc ctg ttt atg 2442
Leu Gly Gln Glu Pro Leu Ala Leu Glu Ser Leu Phe Met
            105                     110

ttg att cag ggc aag gta aaa gta gta gat tgg gaa gag 2481
Leu Ile Gln Gly Lys Val Lys Val Val Asp Trp Glu Glu
115                 120                     125

tct act gaa gtg aca ctc tac ttc ctc tcc ctt ctt gtc 2520
Ser Thr Glu Val Thr Leu Tyr Phe Leu Ser Leu Leu Val
        130                 135                     140

ttt cgg ctc caa aac ctg cct tgt gga atg gag aag cta 2559
Phe Arg Leu Gln Asn Leu Pro Cys Gly Met Glu Lys Leu
                145                     150

ggc agt gac gtt gct act ctc ttt acc cat tcg cat gtc 2598
Gly Ser Asp Val Ala Thr Leu Phe Thr His Ser His Val
    155                 160                     165

gtc tct ctt gtg agt gca gca gcc tgt cta ttg gga cag 2637
Val Ser Leu Val Ser Ala Ala Ala Cys Leu Leu Gly Gln
            170                     175

ctt ggt cag caa ggg gtg acc ttt gac ctc cag ccc atg 2676
Leu Gly Gln Gln Gly Val Thr Phe Asp Leu Gln Pro Met
180                 185                     190

gaa tgg atg gct gca gcc aca cat gcc ttg tct gcc cct 2715
Glu Trp Met Ala Ala Ala Thr His Ala Leu Ser Ala Pro
        195                 200                     205

gca gag ctc ctc act gag gta cag atg gat ctt ggg atg 2754
Ala Glu Leu Leu Thr Glu Val Gln Met Asp Leu Gly Met
                210                     215

gat ggg aag taaagag agaggaactg ggcatttggg ggagcctctg 2800
Asp Gly Lys
    220 221

gaccagagga atgaagaagc aacccacagc cttccctctc aagctactgt 2850

gcctgtgata gccttggaac ttccccgcct gccctcagta ctgacccttt 2900

gaaggaaacc attcgctgcg tcccctggga tccagtggga gataaaatga 2950

attccctggg tttcagcaga catacacatg agttgtgagg tcagagggtt 3000

aaggtttgat aagaaaatga aataagacga cagggaaata ctaggtggga 3050
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attatccctt agccaacatt cctatctgtg ggtgggcgtg gagagtgtat 5050

ctttttttgg ggtgtgtgtg tatatgtgtg tgtgtatgtg tgtgtgtgtt 5100

taatagttct gtttgtaaac tcttttaata aaagttgtgc ctcaccatac 5150

ttgaagctcc caggacaagg gttgagaggc tcaacccctc tttcagcttc 5200

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aa 5252

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Gly Arg Val Tyr Lys Gly Arg Arg Lys Tyr Ser Ala Gln Val Val
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Ala Leu Lys Phe Ile Pro Lys Leu Gly Arg Ser Glu Lys Glu Leu
35 40 45

Arg Asn Leu Gln Arg Glu Ile Glu Ile Met Arg Gly Leu Arg His
50 55 60

Pro Asn Ile Val His Met Leu Asp Ser Phe Glu Thr Asp Lys Glu
65 70 75

Val Val Val Val Thr Asp Tyr Ala Glu Gly Glu Leu Phe Gln Ile
80 85 90

Leu Glu Asp Asp Gly Lys Leu Pro Glu Asp Gln Val Gln Ala Ile

95 100 105

Ala Ala Gln Leu Val Ser Ala Leu Tyr Tyr Leu His Ser His Arg
110 115 120

Ile Leu His Arg Asp Met Lys Pro Gln Asn Ile Leu Leu Ala Lys
125 130 135

Gly Gly Gly Ile Lys Leu Cys Asp Phe Gly Phe Ala Arg Ala Met
140 145 150

Ser Thr Asn Thr Met Val Leu Thr Ser Ile Lys Gly Thr Pro Leu
155 160 165

Tyr Met Ser Pro Glu Leu Val Glu Glu Arg Pro Tyr Asp His Thr
170 175 180

Ala Asp Leu Trp Ser Val Gly Cys Ile Leu Tyr Glu Leu Ala Val
185 190 195

Gly Thr Pro Pro Phe Tyr Ala Thr Ser Ile Phe Gln Leu Val Ser
200 205 210

Leu Ile Leu Lys Asp Pro Val Arg Trp Pro Ser Thr Ile Ser Pro
215 220 225

Cys Phe Lys Asn Phe Leu Gln Gly Leu Leu Thr Lys Asp Pro Arg
230 235 240

Gln Arg Leu Ser Trp Pro Asp Leu Leu Tyr His Pro Phe Ile Ala
245 250 255

Gly His Val Thr Ile Ile Thr Glu Pro Ala Gly Pro Asp Leu Gly
260 265 270

Thr Pro Phe Thr Ser Arg Leu Pro Pro Glu Leu Gln Val Leu Lys
275 280 285

Asp Glu Gln Ala His Arg Leu Ala Pro Lys Gly Asn Gln Ser Arg
290 295 300

Ile Leu Thr Gln Ala Tyr Lys Arg Met Ala Glu Glu Ala Met Gln
305 310 315

Lys Lys His Gln Asn Thr Gly Pro Ala Leu Glu Gln Glu Asp Lys
320 325 330

Thr Ser Lys Val Ala Pro Gly Thr Ala Pro Leu Pro Arg Leu Gly
335 340 345

Ala Thr Pro Gln Glu Ser Ser Leu Leu Ala Gly Ile Leu Ala Ser
350 355 360

Glu Leu Lys Ser Ser Trp Ala Lys Ser Gly Thr Gly Glu Val Pro
365 370 375

Ser Ala Pro Arg Glu Asn Arg Thr Thr Pro Asp Cys Glu Arg Ala

380 385 390

Phe Pro Glu Glu Arg Pro Glu Val Leu Gly Gln Arg Ser Thr Asp
395 400 405

Val Val Asp Leu Glu Asn Glu Glu Pro Asp Ser Asp Asn Glu Trp
410 415 420

Gln His Leu Leu Glu Thr Thr Glu Pro Val Pro Ile Gln Leu Lys
425 430 435

Ala Pro Leu Thr Leu Leu Cys Asn Pro Asp Phe Cys Gln Arg Ile
440 445 450

Gln Ser Gln Leu His Glu Ala Gly Gly Gln Ile Leu Lys Gly Ile
455 460 465

Leu Glu Gly Ala Ser His Ile Leu Pro Ala Phe Arg Val Leu Ser
470 475 480

Ser Leu Leu Ser Ser Cys Ser Asp Ser Val Ala Leu Tyr Ser Phe
485 490 495

Cys Arg Glu Ala Gly Leu Pro Gly Leu Leu Leu Ser Leu Leu Arg
500 505 510

His Ser Gln Glu Ser Asn Ser Leu Gln Gln Gln Ser Trp Tyr Gly
515 520 525

Thr Phe Leu Gln Asp Leu Met Ala Val Ile Gln Ala Tyr Phe Ala
530 535 540

Cys Thr Phe Asn Leu Glu Arg Ser Gln Thr Ser Asp Ser Leu Gln
545 550 555

Val Phe Gln Glu Ala Ala Asn Leu Phe Leu Asp Leu Leu Gly Lys
560 565 570

Leu Leu Ala Gln Pro Asp Asp Ser Glu Gln Thr Leu Arg Arg Asp
575 580 585

Ser Leu Met Cys Phe Thr Val Leu Cys Glu Ala Met Asp Gly Asn
590 595 600

Ser Arg Ala Ile Ser Lys Ala Phe Tyr Ser Ser Leu Leu Thr Thr
605 610 615

Gln Gln Val Val Leu Asp Gly Leu Leu His Gly Leu Thr Val Pro
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Gln Leu Pro Val His Thr Pro Gln Gly Ser Leu Leu Leu Leu Pro
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Cys Arg
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tcgacaagca gggaacaccc aagtagaagc tc 32

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<211> 685
<212> PRT
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20 25 30

Ala Leu Ala Ala Ile Cys Thr Ala Pro Val Gly Leu Pro Asp Cys
35 40 45

Trp Asp Ala Lys Glu Gln Val Cys Trp His Leu Ala Asn Gln Leu
50 55 60

Thr Glu Asp Ser Ser Gln Leu Arg Pro Ser Leu Ile Ser Gly Leu
65 70 75

Gln His Pro Ile Leu Cys Leu His Leu Leu Lys Val Leu Tyr Ser
80 85 90

Cys Cys Leu Val Ser Glu Gly Leu Cys Arg Leu Leu Gly Gln Glu
95 100 105

Pro Leu Ala Leu Glu Ser Leu Phe Met Leu Ile Gln Gly Lys Val
110 115 120

Lys Val Val Asp Trp Glu Glu Ser Thr Glu Val Thr Leu Tyr Phe
125 130 135

Leu Ser Leu Leu Val Phe Arg Leu Gln Asn Leu Pro Cys Gly Met
140 145 150

Glu Lys Leu Gly Ser Asp Val Ala Thr Leu Phe Thr His Ser His
155 160 165

Val Val Ser Leu Val Ser Ala Ala Ala Cys Leu Leu Gly Gln Leu
170 175 180

Gly Gln Gln Gly Val Thr Phe Asp Leu Gln Pro Met Glu Trp Met
185 190 195

Ala Ala Ala Thr His Ala Leu Ser Ala Pro Ala Glu Val Arg Leu
200 205 210

Thr Pro Pro Gly Ser Cys Gly Phe Tyr Asp Gly Leu Leu Ile Leu
215 220 225

Leu Leu Gln Leu Leu Thr Glu Gln Gly Lys Ala Ser Leu Ile Arg
230 235 240

Asp Met Ser Ser Ser Glu Met Trp Thr Val Leu Trp His Arg Phe
245 250 255

Ser Met Val Leu Arg Leu Pro Glu Glu Ala Ser Ala Gln Glu Gly
260 265 270

Glu Leu Ser Leu Ser Ser Pro Pro Ser Pro Glu Pro Asp Trp Thr
275 280 285

Leu Ile Ser Pro Gln Gly Met Ala Ala Leu Leu Ser Leu Ala Met
290 295 300

Ala Thr Phe Thr Gln Glu Pro Gln Leu Cys Leu Ser Cys Leu Ser
305 310 315

Gln His Gly Ser Ile Leu Met Ser Ile Leu Lys His Leu Leu Cys
320 325 330

Pro Ser Phe Leu Asn Gln Leu Arg Gln Ala Pro His Gly Ser Glu
335 340 345

Phe Leu Pro Val Val Val Leu Ser Val Cys Gln Leu Leu Cys Phe
350 355 360

Pro Phe Ala Leu Asp Met Asp Ala Asp Leu Leu Ile Val Val Leu
365 370 375

Ala Asp Leu Arg Asp Ser Glu Val Ala Ala His Leu Leu Gln Val
380 385 390

Cys Cys Tyr His Leu Pro Leu Met Gln Val Glu Leu Pro Ile Ser
395 400 405

Leu Leu Thr Arg Leu Ala Leu Met Asp Pro Thr Ser Leu Asn Gln
410 415 420

Phe Val Asn Thr Val Ser Ala Ser Pro Arg Thr Ile Val Ser Phe
425 430 435

Leu Ser Val Ala Leu Leu Ser Asp Gln Pro Leu Leu Thr Ser Asp
440 445 450

Leu Leu Ser Leu Leu Ala His Thr Ala Arg Val Leu Ser Pro Ser
455 460 465

His Leu Ser Phe Ile Gln Glu Leu Leu Ala Gly Ser Asp Glu Ser
470 475 480

Tyr Arg Pro Leu Arg Ser Leu Leu Gly His Pro Glu Asn Ser Val
485 490 495

Arg Ala His Thr Tyr Arg Leu Leu Gly His Leu Leu Gln His Ser
500 505 510

Met Ala Leu Arg Gly Ala Leu Gln Ser Gln Ser Gly Leu Leu Ser
515 520 525

Leu Leu Leu Leu Gly Leu Gly Asp Lys Asp Pro Val Val Arg Cys
530 535 540

Ser Ala Ser Phe Ala Val Gly Asn Ala Ala Tyr Gln Ala Gly Pro
545 550 555

Leu Gly Pro Ala Leu Ala Ala Ala Val Pro Ser Met Thr Gln Leu
560 565 570

Leu Gly Asp Pro Gln Ala Gly Ile Arg Arg Asn Val Ala Ser Ala
575 580 585

Leu Gly Asn Leu Gly Pro Glu Gly Leu Gly Glu Glu Leu Leu Gln
590 595 600

Cys Glu Val Pro Gln Arg Leu Leu Glu Met Ala Cys Gly Asp Pro
605 610 615

Gln Pro Asn Val Lys Glu Ala Ala Leu Ile Ala Leu Arg Ser Leu
620 625 630

Gln Gln Glu Pro Gly Ile His Gln Val Leu Val Ser Leu Gly Ala
635 640 645

Ser Glu Lys Leu Ser Leu Leu Ser Leu Gly Asn Gln Ser Leu Pro
650 655 660

His Ser Ser Pro Arg Pro Ala Ser Ala Lys His Cys Arg Lys Leu
665 670 675

Ile His Leu Leu Arg Pro Ala His Ser Met
680 685

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<213> Homo sapiens

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Pro Leu Arg Glu Gln Ser Glu Asp Ile Pro Gly Ala Ile Ser Ser
20 25 30

Ala Leu Ala Ala Ile Cys Thr Ala Pro Val Gly Leu Pro Asp Cys
35 40 45

Trp Asp Ala Lys Glu Gln Val Cys Trp His Leu Ala Asn Gln Leu
50 55 60

Thr Glu Asp Ser Ser Gln Leu Arg Pro Ser Leu Ile Ser Gly Leu
65 70 75

Gln His Pro Ile Leu Cys Leu His Leu Leu Lys Val Leu Tyr Ser
80 85 90

Cys Cys Leu Val Ser Glu Gly Leu Cys Arg Leu Leu Gly Gln Glu
95 100 105

Pro Leu Ala Leu Glu Ser Leu Phe Met Leu Ile Gln Gly Lys Val
110 115 120

Lys Val Val Asp Trp Glu Glu Ser Thr Glu Val Thr Leu Tyr Phe
125 130 135

Leu Ser Leu Leu Val Phe Arg Leu Gln Asn Leu Pro Cys Gly Met
140 145 150

Glu Lys Leu Gly Ser Asp Val Ala Thr Leu Phe Thr His Ser His
155 160 165

Val Val Ser Leu Val Ser Ala Ala Ala Cys Leu Leu Gly Gln Leu
170 175 180

Gly Gln Gln Gly Val Thr Phe Asp Leu Gln Pro Met Glu Trp Met
185 190 195

Ala Ala Ala Thr His Ala Leu Ser Ala Pro Ala Glu Leu Leu Thr
200 205 210

Glu Val Gln Met Asp Leu Gly Met Asp Gly Lys
215 220 221

<210> 23
<211> 795
<212> PRT

<213> Drosophila virilis

<400> 23

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Gly Cys Val Tyr Lys Ala Gln Arg Arg Asp Asp Asp Lys Val Val
            20                  25                  30

Ala Ile Lys Val Ile Ser Lys Arg Gly Arg Ser Asn Arg Glu Leu
        35                  40                  45

Lys Asn Leu Arg Arg Glu Cys Asp Ile Gln Ala Arg Leu Lys His
    50                  55                  60

Pro His Val Ile Glu Met Val Glu Ser Phe Glu Ser Lys Phe Asp
65                  70                  75

Leu Phe Val Val Thr Glu Phe Ala Leu Met Asp Leu His Arg Tyr
                80                  85                  90

Leu Ser Phe Asn Gly Ala Met Pro Glu Glu His Ala Gln Arg Val
            95                  100                 105

Val Cys His Leu Val Ser Ala Leu Tyr Tyr Leu His Ser Asn Arg
        110                 115                 120

Ile Leu His Arg Asp Leu Lys Pro Gln Asn Val Leu Leu Asp Lys
    125                 130                 135

Asn Met His Ala Lys Leu Cys Asp Phe Gly Leu Ala Arg Asn Met
140                 145                 150

Thr Met Gly Thr His Val Leu Thr Ser Ile Lys Gly Thr Pro Leu
                155                 160                 165

Tyr Met Ala Pro Glu Leu Leu Ala Glu Gln Pro Tyr Asp His Gln
            170                 175                 180

Ala Asp Met Trp Ser Leu Gly Cys Ile Ala Tyr Glu Ser Met Ala
        185                 190                 195

Gly Gln Pro Pro Phe Cys Ala Thr Ser Ile Leu His Leu Val Lys
    200                 205                 210

Leu Ile Lys His Glu Asp Val Lys Trp Pro Ser Thr Leu Ser Ser
215                 220                 225

Glu Cys Arg Ser Phe Leu Gln Gly Leu Leu Glu Lys Asp Pro Ser
                230                 235                 240

Met Arg Ile Ser Trp Thr Gln Leu Leu Cys His Pro Phe Val Glu
            245                 250                 255

Gly Lys Leu Tyr Ile Ala Glu Val Gln Ala Ala Gln Thr Ser Pro
        260                 265                 270
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Phe Ile Asn Pro Gln Leu Ala Lys Asp Thr Lys Lys Ser Gln Gln
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Leu Arg His Val Gly Ala Asp Leu Gly Asp Val Leu Ala Ala Leu
290 295 300

Lys Leu Ser Asp Val Ala Asn Glu Asn Leu Ser Thr Ser Arg Asp
305 310 315

Ser Ile Asn Ala Ile Ala Pro Ser Asp Ile Glu Gln Leu Glu Thr
320 325 330

Asp Val Glu Asp Asn Val His Arg Leu Ile Val Pro Phe Ala Asp
335 340 345

Ile Ser Tyr Arg Glu Leu Pro Cys Gly Thr Ala Ala Ala Ala Arg
350 355 360

Arg Ala Gly Ala Met Pro Leu Ile Asn Ser Gln Thr Cys Phe Val
365 370 375

Ser Gly Asn Ser Asn Met Ile Leu Asn His Leu Asn Asp Asn Phe
380 385 390

Ala Ile Glu Ala Pro Ala Ser Ser Ala Thr Lys Ser Met Lys Ser
395 400 405

Lys Leu Lys Leu Ala Leu Asn Ile Lys Gln Ser Arg Ser Lys Asp
410 415 420

Leu Glu Lys Arg Lys Leu Ser Gln Asn Leu Asp Asn Phe Ser Leu
425 430 435

Arg Leu Gly Gln Ser Ile Asp Ile Glu Val Gln Arg Lys Thr Thr
440 445 450

Glu Met Leu Thr Gln Gln Ser Gln Ala Gln Gln Leu Gln Asp Arg
455 460 465

Lys Thr Gln Gln Leu Lys Gln Ser Met His Ser Thr Asn Asp Glu
470 475 480

Lys Leu Ser Ser Asp Asn Ser Pro Pro Cys Leu Leu Pro Gly Trp
485 490 495

Asp Ser Cys Asp Glu Ser Gln Ser Pro Pro Ile Glu Asn Asp Glu
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Trp Leu Ala Phe Leu His Arg Ser Ile Gln Glu Leu Leu Asp Gly
515 520 525

Glu Phe Asp Ser Leu Lys Gln His Asn Leu Val Ser Ile Ile Val
530 535 540

Ala Pro Leu Arg Asn Ser Lys Ala Ile Pro Lys Val Leu Gln Ser
545 550 555

Val Ala Gln Leu Leu Ser Leu Pro Phe Val Leu Ala Glu Gln His
560 565 570

Leu Val Ala Glu Ala Ile Lys Gly Val Tyr Ile Asp Val Lys Leu
575 580 585

Val Pro Asn Leu Met Tyr Ala Cys Lys Leu Leu Leu Ser Gln Arg
590 595 600

His Leu Thr Asp Ser Ala Ala Ser Leu Pro Ala Gly Thr Gly Val
605 610 615

Ser Leu Ser Arg Thr Val Arg Ser Cys Ser Asp Leu Ser Ala Glu
620 625 630

Glu Met Ser Thr Ala Cys Ser Leu Tyr Glu Leu Val Cys His Leu
635 640 645

Val His Gln Gln Gln Gln Phe Leu Thr Gln Phe Cys Asp Ala Val
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Ala Ile Leu Ala Val Asn Asp Met Phe Ile Asn Phe Leu Thr His
665 670 675

Asp Phe Lys Asp Ser Arg Pro Val Arg Leu Ala Ser Cys Met Leu
680 685 690

Ala Leu Phe Cys Cys Val Leu Arg Glu Leu Pro Glu Asn Ala Glu
695 700 705

Leu Val Glu Lys Ile Val Phe Asp Ser Arg Leu Gln Leu Ala Val
710 715 720

Leu Leu Gln Ser Arg His His Leu Leu Arg Gln Arg Ala Cys Gln
725 730 735

Met Leu Leu Leu Leu Ala Arg Phe Ser Leu Arg Gly Val Gln Cys
740 745 750

Ile Trp Ser Gly Glu Leu Lys Ser Ala Leu Gln Ala Trp Pro Met
755 760 765

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Asp Glu Leu Ser Gln Phe Ser Phe Phe Val Ala Gln Ala Thr Ala
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<212> PRT
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35 40 45

Arg Asn Leu Gln Arg Glu Ile Glu Ile Met Arg Gly Leu Arg His
50 55 60

Pro Asn Ile Val His Met Leu Asp Ser Phe Glu Thr Asp Lys Glu
65 70 75

Val Val Val Val Thr Asp Tyr Ala Glu Gly Glu Leu Phe Gln Ile
80 85 90

Leu Glu Asp Asp Gly Lys Leu Pro Glu Asp Gln Val Gln Ala Ile
95 100 105

Ala Ala Gln Leu Val Ser Ala Leu Tyr Tyr Leu His Ser His Arg
110 115 120

Ile Leu His Arg Asp Met Lys Pro Gln Asn Ile Leu Leu Ala Lys
125 130 135

Gly Gly Gly Ile Lys Leu Cys Asp Phe Gly Phe Ala Arg Ala Met
140 145 150

Ser Thr Asn Thr Met Val Leu Thr Ser Ile Lys Gly Thr Pro Leu
155 160 165

Tyr Met Ser Pro Glu Leu Val Glu Glu Arg Pro Tyr Asp His Thr
170 175 180

Ala Asp Leu Trp Ser Val Gly Cys Ile Leu Tyr Glu Leu Ala Val
185 190 195

Gly Thr Pro Pro Phe Tyr Ala Thr Ser Ile Phe Gln Leu Val Ser
200 205 210

Leu Ile Leu Lys Asp Pro Val Arg Trp Pro Ser Thr Ile Ser Pro
215 220 225

Cys Phe Lys Asn Phe Leu Gln Gly Leu Leu Thr Lys Asp Pro Arg
230 235 240

Gln Arg Leu Ser Trp Pro Asp Leu Leu Tyr His Pro Phe Ile Ala
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Gly His Val Thr Ile
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Ala Leu Arg Phe Ile Pro Lys Leu Gly Arg Ser Glu Lys Glu Leu
35 40 45

Arg Asn Leu Gln Arg Glu Ile Glu Ile Met Arg Gly Leu Arg His
50 55 60

Pro Asn Ile Val His Met Leu Asp Ser Phe Glu Thr Asp Lys Glu
65 70 75

Val Val Val Val Thr Asp Tyr Ala Glu Gly Glu Leu Phe Gln Ile
80 85 90

Leu Glu Asp Asp Gly Lys Leu Pro Glu Asp Gln Val Gln Ala Ile
95 100 105

Ala Ala Gln Leu Val Ser Ala Leu Tyr Tyr Leu His Ser His Arg
110 115 120

Ile Leu His Arg Asp Met Lys Pro Gln Asn Ile Leu Leu Ala Lys
125 130 135

Gly Gly Gly Ile Lys Leu Cys Asp Phe Gly Phe Ala Arg Ala Met
140 145 150

Ser Thr Asn Thr Met Val Leu Thr Ser Ile Lys Gly Thr Pro Leu
155 160 165

Tyr Met Ser Pro Glu Leu Val Glu Glu Arg Pro Tyr Asp His Thr
170 175 180

Ala Asp Leu Trp Ser Val Gly Cys Ile Leu Tyr Glu Leu Ala Val
185 190 195

Gly Thr Pro Pro Phe Tyr Ala Thr Ser Ile Phe Gln Leu Val Ser
200 205 210

Leu Ile Leu Lys Asp Pro Val Arg Trp Pro Ser Thr Ile Ser Pro
215 220 225

Cys Phe Lys Asn Phe Leu Gln Gly Leu Leu Thr Lys Asp Pro Arg
230 235 240

Gln Arg Leu Ser Trp Pro Asp Leu Leu Tyr His Pro Phe Ile Ala
245 250 255

Gly His Val Thr Ile Ile Thr Glu Pro Ala Gly Pro Asp Leu Gly
260 265 270

Thr Pro Phe Thr Ser Arg Leu Pro Pro Glu Leu Gln Val Leu Lys
275 280 285

Asp Glu Gln Ala His Arg Leu Ala Pro Lys Gly Asn Gln Ser Arg
290 295 300

Ile Leu Thr Gln Ala Tyr Lys Arg Met Ala Glu Glu Ala Met Gln
305 310 315

Lys Lys His Gln Asn Thr Gly Pro Ala Leu Glu Gln Glu Asp Lys
320 325 330

Thr Ser Lys Val Ala Pro Gly Thr Ala Pro Leu Pro Arg Leu Gly
335 340 345

Ala Thr Pro Gln Glu Ser Ser Leu Leu Ala Gly Ile Leu Ala Ser
350 355 360

Glu Leu Lys Ser Ser Trp Ala Lys Ser Gly Thr Gly Glu Val Pro
365 370 375

Ser Ala Pro Arg Glu Asn Arg Thr Thr Pro Asp Cys Glu Arg Ala
380 385 390

Phe Pro Glu Glu Arg Pro Glu Val Leu Gly Gln Arg Ser Thr Asp
395 400 405

Val Val Asp Leu Glu Asn Glu Glu Pro Asp Ser Asp Asn Glu Trp
410 415 420

Gln His Leu Leu Glu Thr Thr Glu Pro Val Pro Ile Gln Leu Lys
425 430 435

Ala Pro Leu Thr Leu Leu Cys Asn Pro Asp Phe Cys Gln Arg Ile
440 445 450

Gln Ser Gln Leu His Glu Ala Gly Gly Gln Ile Leu Lys Gly Ile
455 460 465

Leu Glu Gly Ala Ser His Ile Leu Pro Ala Phe Arg Val Leu Ser
470 475 480

Ser Leu Leu Ser Ser Cys Ser Asp Ser Val Ala Leu Tyr Ser Phe
485 490 495

Cys Arg Glu Ala Gly Leu Pro Gly Leu Leu Leu Ser Leu Leu Arg
500 505 510

His Ser Gln Glu Ser Asn Ser Leu Gln Gln Gln Ser Trp Tyr Gly
515 520 525

Thr Phe Leu Gln Asp Leu Met Ala Val Ile Gln Ala Tyr Phe Ala
530 535 540

Cys Thr Phe Asn Leu Glu Arg Ser Gln Thr Ser Asp Ser Leu Gln
545 550 555

Val Phe Gln Glu Ala Ala Asn Leu Phe Leu Asp Leu Leu Gly Lys
560 565 570

Leu Leu Ala Gln Pro Asp Asp Ser Glu Gln Thr Leu Arg Arg Asp
575 580 585

Ser Leu Met Cys Phe Thr Val Leu Cys Glu Ala Met Asp Gly Asn
590 595 600

Ser Arg Ala Ile Ser Lys Ala Phe Tyr Ser Ser Leu Leu Thr Thr
605 610 615

Gln Gln Val Val Leu Asp Gly Leu Leu His Gly Leu Thr Val Pro
620 625 630

Gln Leu Pro Val His Thr Pro Gln Gly Ala Pro Gln Val Ser Gln
635 640 645

Pro Leu Arg Glu Gln Ser Glu Asp Ile Pro Gly Ala Ile Ser Ser
650 655 660

Ala Leu Ala Ala Ile Cys Thr Ala Pro Val Gly Leu Pro Asp Cys
665 670 675

Trp Asp Ala Lys Glu Gln Val Cys Trp His Leu Ala Asn Gln Leu
680 685 690

Thr Glu Asp Ser Ser Gln Leu Arg Pro Ser Leu Ile Ser Gly Leu
695 700 705

Gln His Pro Ile Leu Cys Leu His Leu Leu Lys Val Leu Tyr Ser
710 715 720

Cys Cys Leu Val Ser Glu Gly Leu Cys Arg Leu Leu Gly Gln Glu
725 730 735

Pro Leu Ala Leu Glu Ser Leu Phe Met Leu Ile Gln Gly Lys Val
740 745 750

Lys Val Val Asp Trp Glu Glu Ser Thr Glu Val Thr Leu Tyr Phe
755 760 765

Leu Ser Leu Leu Val Phe Arg Leu Gln Asn Leu Pro Cys Gly Met
770 775 780

Glu Lys Leu Gly Ser Asp Val Ala Thr Leu Phe Thr His Ser His
785 790 795

Val Val Ser Leu Val Ser Ala Ala Ala Cys Leu Leu Gly Gln Leu
800 805 810

Gly Gln Gln Gly Val Thr Phe Asp Leu Gln Pro Met Glu Trp Met
815 820 825

Ala Ala Ala Thr His Ala Leu Ser Ala Pro Ala Glu Val Arg Leu
830 835 840

Thr Pro Pro Gly Ser Cys Gly Phe Tyr Asp Gly Leu Leu Ile Leu
845 850 855

Leu Leu Gln Leu Leu Thr Glu Gln Gly Lys Ala Ser Leu Ile Arg
860 865 870

Asp Met Ser Ser Ser Glu Met Trp Thr Val Leu Trp His Arg Phe
875 880 885

Ser Met Val Leu Arg Leu Pro Glu Glu Ala Ser Ala Gln Glu Gly
890 895 900

Glu Leu Ser Leu Ser Ser Pro Pro Ser Pro Glu Pro Asp Trp Thr
905 910 915

Leu Ile Ser Pro Gln Gly Met Ala Ala Leu Leu Ser Leu Ala Met
920 925 930

Ala Thr Phe Thr Gln Glu Pro Gln Leu Cys Leu Ser Cys Leu Ser
935 940 945

Gln His Gly Ser Ile Leu Met Ser Ile Leu Lys His Leu Leu Cys
950 955 960

Pro Ser Phe Leu Asn Gln Leu Arg Gln Ala Pro His Gly Ser Glu
965 970 975

Phe Leu Pro Val Val Val Leu Ser Val Cys Gln Leu Leu Cys Phe
980 985 990

Pro Phe Ala Leu Asp Met Asp Ala Asp Leu Leu Ile Val Val Leu
995 1000 1005

Ala Asp Leu Arg Asp Ser Glu Val Ala Ala His Leu Leu Gln Val
1010 1015 1020

Cys Cys Tyr His Leu Pro Leu Met Gln Val Glu Leu Pro Ile Ser
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Leu Leu Thr Arg Leu Ala Leu Met Asp Pro Thr Ser Leu Asn Gln
1040 1045 1050

Phe Val Asn Thr Val Ser Ala Ser Pro Arg Thr Ile Val Ser Phe
1055 1060 1065

Leu Ser Val Ala Leu Leu Ser Asp Gln Pro Leu Leu Thr Ser Asp
1070 1075 1080

Leu Leu Ser Leu Leu Ala His Thr Ala Arg Val Leu Ser Pro Ser
1085 1090 1095

His Leu Ser Phe Ile Gln Glu Leu Leu Ala Gly Ser Asp Glu Ser
1100 1105 1110

Tyr Arg Pro Leu Arg Ser Leu Leu Gly His Pro Glu Asn Ser Val
1115 1120 1125

Arg Ala His Thr Tyr Arg Leu Leu Gly His Leu Leu Gln His Ser
1130 1135 1140

Met Ala Leu Arg Gly Ala Leu Gln Ser Gln Ser Gly Leu Leu Ser
1145 1150 1155

Leu Leu Leu Leu Gly Leu Gly Asp Lys Asp Pro Val Val Arg Cys
1160 1165 1170

Ser Ala Ser Phe Ala Val Gly Asn Ala Ala Tyr Gln Ala Gly Pro
1175 1180 1185

Leu Gly Pro Ala Leu Ala Ala Ala Val Pro Ser Met Thr Gln Leu
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Leu Gly Asp Pro Gln Ala Gly Ile Arg Arg Asn Val Ala Ser Ala
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Leu Gly Asn Leu Gly Pro Glu Gly Leu Gly Glu Glu Leu Leu Gln
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Cys Glu Val Pro Gln Arg Leu Leu Glu Met Ala Cys Gly Asp Pro
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Gln Pro Asn Val Lys Glu Ala Ala Leu Ile Ala Leu Arg Ser Leu
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Gln Gln Glu Pro Gly Ile His Gln Val Leu Val Ser Leu Gly Ala
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Ser Glu Lys Leu Ser Leu Leu Ser Leu Gly Asn Gln Ser Leu Pro
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His Ser Ser Pro Arg Pro Ala Ser Ala Lys His Cys Arg Lys Leu
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Ile His Leu Leu Arg Pro Ala His Ser Met
1310 1315

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<213> Drosophila virilis

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<212> PRT
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<400> 27

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                20                  25                  30

Arg Leu Ala Pro Lys Gly Asn Gln Ser Arg Ile Leu Thr Gln Ala
                35                  40                  45

Tyr Lys Arg Met Ala Glu Glu Ala Met Gln Lys Lys His Gln Asn
                50                  55                  60

Thr Gly Pro Ala Leu Glu Gln Glu Asp Lys Thr Ser Lys Val Ala
                65                  70                  75

Pro Gly Thr Ala Pro Leu Pro Arg Leu Gly Ala Thr Pro Gln Glu
                80                  85                  90

Ser Ser Leu Leu Ala Gly Ile Leu Ala Ser Glu Leu Lys Ser Ser
                95                  100                 105

Trp Ala Lys Ser Gly Thr Gly Glu Val Pro Ser Ala Pro Arg Glu
                110                 115                 120

Asn Arg Thr Thr Pro Asp Cys Glu Arg Ala Phe Pro Glu Glu Arg
                125                 130                 135

Pro Glu Val Leu Gly Gln Arg Ser Thr Asp Val Val Asp Leu Glu
                140                 145                 150

Asn Glu Glu Pro Asp Ser Asp Asn Glu Trp Gln His Leu Leu Glu
                155                 160                 165

Thr Thr Glu Pro Val Pro Ile Gln Leu Lys Ala Pro Leu Thr Leu
                170                 175                 180

Leu Cys Asn Pro Asp Phe Cys Gln Arg Ile Gln Ser Gln Leu His
                185                 190                 195

Glu Ala Gly Gly Gln Ile Leu Lys Gly Ile Leu Glu Gly Ala Ser
                200                 205                 210

His Ile Leu Pro Ala Phe Arg Val Leu Ser Ser Leu Leu Ser Ser
                215                 220                 225

Cys Ser Asp Ser Val Ala Leu Tyr Ser Phe Cys Arg Glu Ala Gly
                230                 235                 240

Leu Pro Gly Leu Leu Leu Ser Leu Leu Arg His Ser Gln Glu Ser
                245                 250                 255

Asn Ser Leu Gln Gln Gln Ser Trp Tyr Gly Thr Phe Leu Gln Asp
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260 265 270

Leu Met Ala Val Ile Gln Ala Tyr Phe Ala Cys Thr Phe Asn Leu
275 280 285

Glu Arg Ser Gln Thr Ser Asp Ser Leu Gln Val Phe Gln Glu Ala
290 295 300

Ala Asn Leu Phe Leu Asp Leu Leu Gly Lys Leu Leu Ala Gln Pro
305 310 315

Asp Asp Ser Glu Gln Thr Leu Arg Arg Asp Ser Leu Met Cys Phe
320 325 330

Thr Val Leu Cys Glu Ala Met Asp Gly Asn Ser Arg Ala Ile Ser
335 340 345

Lys Ala Phe Tyr Ser Ser Leu Leu Thr Thr Gln Gln Val Val Leu
350 355 360

Asp Gly Leu Leu His Gly Leu Thr Val Pro Gln Leu Pro Val His
365 370 375

Thr Pro Gln Gly Ala Pro Gln Val Ser Gln Pro Leu Arg Glu Gln
380 385 390

Ser Glu Asp Ile Pro Gly Ala Ile Ser Ser Ala Leu Ala Ala Ile
395 400 405

Cys Thr Ala Pro Val Gly Leu Pro Asp Cys Trp Asp Ala Lys Glu
410 415 420

Gln Val Cys Trp His Leu Ala Asn Gln Leu Thr Glu Asp Ser Ser
425 430 435

Gln Leu Arg Pro Ser Leu Ile Ser Gly Leu Gln His Pro Ile Leu
440 445 450

Cys Leu His Leu Leu Lys Val Leu Tyr Ser Cys Cys Leu Val Ser
455 460 465

Glu Gly Leu Cys Arg Leu Leu Gly Gln Glu Pro Leu Ala Leu Glu
470 475 480

Ser Leu Phe Met Leu Ile Gln Gly Lys Val Lys Val Val Asp Trp
485 490 495

Glu Glu Ser Thr Glu Val Thr Leu Tyr Phe Leu Ser Leu Leu Val
500 505 510

Phe Arg Leu Gln Asn Leu Pro Cys Gly Met Glu Lys Leu Gly Ser
515 520 525

Asp Val Ala Thr Leu Phe Thr His Ser His Val Val Ser Leu Val
530 535 540

Ser Ala Ala Ala Cys Leu Leu Gly Gln Leu Gly Gln Gln Gly Val

545 550 555

Thr Phe Asp Leu Gln Pro Met Glu Trp Met Ala Ala Ala Thr His
560 565 570

Ala Leu Ser Ala Pro Ala Glu Val Arg Leu Thr Pro Pro Gly Ser
575 580 585

Cys Gly Phe Tyr Asp Gly Leu Leu Ile Leu Leu Leu Gln Leu Leu
590 595 600

Thr Glu Gln Gly Lys Ala Ser Leu Ile Arg Asp Met Ser Ser Ser
605 610 615

Glu Met Trp Thr Val Leu Trp His Arg Phe Ser Met Val Leu Arg
620 625 630

Leu Pro Glu Glu Ala Ser Ala Gln Glu Gly Glu Leu Ser Leu Ser
635 640 645

Ser Pro Pro Ser Pro Glu Pro Asp Trp Thr Leu Ile Ser Pro Gln
650 655 660

Gly Met Ala Ala Leu Leu Ser Leu Ala Met Ala Thr Phe Thr Gln
665 670 675

Glu Pro Gln Leu Cys Leu Ser Cys Leu Ser Gln His Gly Ser Ile
680 685 690

Leu Met Ser Ile Leu Lys His Leu Leu Cys Pro Ser Phe Leu Asn
695 700 705

Gln Leu Arg Gln Ala Pro His Gly Ser Glu Phe Leu Pro Val Val
710 715 720

Val Leu Ser Val Cys Gln Leu Leu Cys Phe Pro Phe Ala Leu Asp
725 730 735

Met Asp Ala Asp Leu Leu Ile Val Val Leu Ala Asp Leu Arg Asp
740 745 750

Ser Glu Val Ala Ala His Leu Leu Gln Val Cys Cys Tyr His Leu
755 760 765

Pro Leu Met Gln Val Glu Leu Pro Ile Ser Leu Leu Thr Arg Leu
770 775 780

Ala Leu Met Asp Pro Thr Ser Leu Asn Gln Phe Val Asn Thr Val
785 790 795

Ser Ala Ser Pro Arg Thr Ile Val Ser Phe Leu Ser Val Ala Leu
800 805 810

Leu Ser Asp Gln Pro Leu Leu Thr Ser Asp Leu Leu Ser Leu Leu
815 820 825

Ala His Thr Ala Arg Val Leu Ser Pro Ser His Leu Ser Phe Ile

830 835 840

Gln Glu Leu Leu Ala Gly Ser Asp Glu Ser Tyr Arg Pro Leu Arg
845 850 855

Ser Leu Leu Gly His Pro Glu Asn Ser Val Arg Ala His Thr Tyr
860 865 870

Arg Leu Leu Gly His Leu Leu Gln His Ser Met Ala Leu Arg Gly
875 880 885

Ala Leu Gln Ser Gln Ser Gly Leu Leu Ser Leu Leu Leu Leu Gly
890 895 900

Leu Gly Asp Lys Asp Pro Val Val Arg Cys Ser Ala Ser Phe Ala
905 910 915

Val Gly Asn Ala Ala Tyr Gln Ala Gly Pro Leu Gly Pro Ala Leu
920 925 930

Ala Ala Ala Val Pro Ser Met Thr Gln Leu Leu Gly Asp Pro Gln
935 940 945

Ala Gly Ile Arg Arg Asn Val Ala Ser Ala Leu Gly Asn Leu Gly
950 955 960

Pro Glu Gly Leu Gly Glu Glu Leu Leu Gln Cys Glu Val Pro Gln
965 970 975

Arg Leu Leu Glu Met Ala Cys Gly Asp Pro Gln Pro Asn Val Lys
980 985 990

Glu Ala Ala Leu Ile Ala Leu Arg Ser Leu Gln Gln Glu Pro Gly
995 1000 1005

Ile His Gln Val Leu Val Ser Leu Gly Ala Ser Glu Lys Leu Ser
1010 1015 1020

Leu Leu Ser Leu Gly Asn Gln Ser Leu Pro His Ser Ser Pro Arg
1025 1030 1035

Pro Ala Ser Ala Lys His Cys Arg Lys Leu Ile His Leu Leu Arg
1040 1045 1050

Pro Ala His Ser Met
1055

INTERNATIONAL SEARCH REPORT

In ational Application No
PCT/US 99/04112

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/54 C12N9/12 C12N1/19 C12N1/21 C12N5/10
C12N15/62 C07K19/00 C12N15/11 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	BLANCHET-TOURNIER M.-F. ET AL.: "The segment-polarity gene 'fused' is highly conserevd in Drosophila." GENE, vol. 161, 1995, pages 157-162, XP002109761 cited in the application the whole document ---	1-24, 27-29
A	THÉROND P. P. ET AL.: "Phosphorylation of the fused protein kinase in response to signaling from hedgehog." PROC. NATL. ACAD. SCI. U.S.A., vol. 93, April 1996 (1996-04), pages 4224-4228, XP002109762 cited in the application the whole document ---	1-24, 27-29

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[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 July 1999	04/08/1999
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Mandl, B

2

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/04112

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
A	HAMMERSCHMIDT M. ET AL.: "The world accoring to hedgehog." TRENDS IN GENETICS, vol. 13, no. 1, January 1997 (1997-01), pages 14-21, XP002109763 page 16, right-hand column, line 31 - page 18, right-hand column, line 46 page 19, left-hand column, last paragraph -----	1-24, 27-29

International application No.
PCT/US 99/04112

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 25,26
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 99 /04112

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 25,26

Present claims 22-26 relate to a compound defined by reference to a desirable characteristic or property, namely its antagonistic or agonistic effect on 'fused', respectively.

The claims cover all compounds having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such compounds. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the compound by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to antisense oligonucleotides which can be obtained from the specific sequence as it is presented by SEQ.ID.1 by using general methods known to the person skilled in the art. Consequently, claims 22 and 23 were searched partially and claim 24 was searched completely.
Moreover, there is an inconsistency between claim 24 referring to an 'antisense nucleotide' and page 4, line 21, referring to an 'antisense oligonucleotide'. Due to the fact that only the term 'antisense oligonucleotide' makes sense in the context of claim 24, claim 24 was read as referring to an 'antisense oligonucleotide'.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.